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A COMPLETION REPORT FOR THE GRANT ENTITLED "USE OF MYXOCOCCUS PCO2 TO CONTROL AQUATIC ALGAE"

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United States
Department of the Interior

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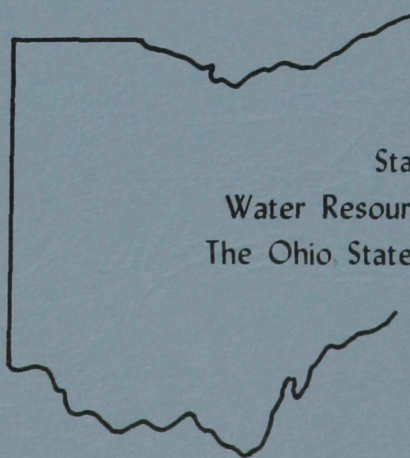

State of Ohio
Water Resources Center
The Ohio State University

A COMPLETION REPORT FOR THE GRANT ENTITLED

"USE OF MYXOCOCCUS PCO2 TO CONTROL AQUATIC ALGAE"

by

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RESEARCH ON WHICH THIS REPORT IS BASED WAS FINANCED IN PART BY THE U. S. DEPARTMENT OF INTERIOR AS AUTHORIZED BY THE WATER RESOURCES AND DEVELOPMENT ACT OF 1978, (P. L. 95-467).

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ACKNOWLEDGEMENTS

a) This project was primarily supported by Matching Grant #B-086-OHIO from the Office of Water Research and Technology, U. S. Department of Interior. Appreciation is expressed to Dr. Robert C. Steifel, Director, and the Ohio Water Resources Center, located at the Ohio State University, for their administration of this grant program.

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PROJECT OBJECTIVES

The long-range goal for this research is to develop an efficient means for employing bacteria to control aqueous populations of algae in natural water resources. The practical applicaton of such algal control requires the completion of the following specific objectives:

1. To describe the mechanisms of destruction of aquatic algal species by the bacterial isolate Myxococcus xanthus strain PC02 on:
   a) solid surfaces
   b) aqueous suspensions

2. To define optimum conditions for the control of various algal species by the Myxococcus PC02.

3. To study population kinetics of both the host and parasite during specific conditions of interaction with respect to parameters such as:
   a) time
   b) temperature
   c) inoculum size
   d) host/parasite ratio
   e) effect of additional bacterial flora

4. To study the effect that Myxococcus PC02 has on stable aquatic microcosms.

BACKGROUND

This research tries to blend bacterial physiology and electron microscopy in order to solve a problem in applied ecology - how to utilize bacteria to control populations of bluegreen algae. Soil runoff has been shown to be a major cause of the detriment of water quality because of the nutrients that are carried into the water. As the nitrates and phosphates accumulate, the waters become eutrophic and the cyanobacterial (bluegreen algae) populations increase to undesirable levels, causing taste and odor problems, poor aesthetics, and occasionally toxic gastroenteritis if ingested. Most significantly, as these microorganisms die and decay, the dissolved oxygen in the water is depleted, leading to a total disruption of the desired ecological system. Management of such problems should use a combination of new land use practices and water quality control measures. Biological control of these bluegreen algal populations by use of a potent bacterial antagonist would prevent the buildup of algae levels in eutrophic waters, thereby preventing or reducing the above problems. Myxococcus PCO2 appears to be a bacterium well suited to employment as such a control system.

The following summarizes the information available concerning bacteriolysis of cyanobacteria and algae. More details are available in reviews written by this author (Burnham, et al., 1981; Burnham and Fraleigh, 1984) and by Professors Stewart and Daft (1977).

Many bacteria have been shown to lyse species of algae. A vibrio was reported to attach to several species of the green algal genus, Chlorella by attaching to the cells and then lysing them (Mamkaeva, 1966; Starr and Seidler, 1971). The mechanisms for this activity are not resolved. A report described an antibiotic substance produced by the genus Cellvibrio, that is capable of lysing vegetative cells of the bluegreen algae, Anabaena inaequalis (Granhall and Berg, 1972). It is particularly interesting that a bacterial protease had no effect on the algicide activity nor did boiling for 15

minutes. While pepsin had no effect, papain was two-thirds inhibitory. Molecular weight determinations by filtration support the above data indicating that the weight was between 1,000 and 10,000. The activity of this substance is apparently grown-dependent as darkness inhibited its lytic effect. This lysis occurred in liquid systems and the authors suggested that the lytic substance might play a role in the algal control in natural ecosystems (Granhall and Berg, 1972).

Coder and Starr (1978) recently described the algal antagonistic bacterium Bdellovibrio chlorellavorus to be effective in killing and ingesting cells of the green algal genus Chlorella. These chlorellavorus bacteria attach to the surface of the algae and penetrate the wall layers with a spike-like mechanism. The bacteria appear to be obligate symbiotes as they are unable to grow on non-living cells or on other heterotrophic nutrients.

Berland et al. (1972) looked at the toxicity of about 50 strains of bacteria for a variety of marine algae. Pseudomonas aeruginosa was found to be particularly inhibitory to Tetraselmis striata, a member of the Prasinophyceae. These authors concluded that it was not possible to state that bacteria or their byproducts are important in determining algal-bacteria relationships in the oceans.

Safferman and Morris (1962) demonstrated that actinomycete filtrates had considerable inhibitory activity against several strains of bluegreen algae. This work resulted in the suggestion that these antibiotic substances could be used as algicides. Sladekova and Sladek (1968) supported this idea of using bacterial secretion of antibiotics in the environment to control algae.

Daft et al. (1984) reports that species of Actinoplanaceae secrete active products that are effective in lysing large numbers of species of both green and bluegreen algae. Similarly, a Bacillus brevis strain was shown to produce an extracellular product that caused lysis of both several bluegreen algal species and several bacterial species (Reim et al., 1974). This non-enzymatic

substance was quite heat-stable, of low molecular weight and could possibly be identified as an antibiotic similar to Gramicidin S. Reim et al., (1974) indicate that the utility of an anti-algal antibiotic control system may be questionable due to the inability to achieve sufficient concentrations of the inhibitor in the general environment.

Investigations of a unique parasitic bacterium Bdellovibrio bacteriovorus have demonstrated the effectiveness of this small microorganism in destroying populations of host bacteria (Burnham, Hashimoto and Conti, 1968; Starr and Seidler, 1971); Rittenberg and Thomashow, 1979) and cyanobacteria (Burnham, et al., 1976; Burnham and Sun, 1977; Burnham, 1975, 1977). Unfortunately the anticyanobacterial effect requires unnaturally high concentrations of protein to be available to the bdellovibrio to stimulate production of the inhibiting and lytic factors. For this reason my laboratory has not continued research on bdellovibrio/cyanobacterial interactions.

Algal Lysis by Members of the Myxobacteriales and Cytophagales

Stewart and Brown (1969) isolated a Cytophaga which formed plaques on both green and bluegreen algae. These authors indicated the lysis of the algae to be extracellular, but the exact cause of lysis was not described.

Wu et al., (1968) indicated that an unidentified myxobacterium was capable of lysing in liquid culture a strain of Lyngbya and five other bluegreen species. The authors indicated that lysis was associated with a slow "clumpy" growth of the myxobacterium and the production of a lysin.

Shilo (1970) isolated a myxobacter (designated FP-1) that lysed viable vegetative cells of many unicellular and filamentous bluegreen algae. Lysis in liquid cultures was prevented when the algal cultures were shaken. Light microscopy demonstrated that algal lysis only occurred upon polar attachment of the myxobacter to the algal cell. Detection of excreted lytic enzymes was unsuccessful, suggesting that the lytic enzymes may be bound to the surface of the myxobacter.

Five algicidal non-fruiting myxobacteria were described by Stewart and Brown (1971) to have a unigformly high G+C ratio of approximately 70 mole percent. All of these organisms were effective in lysing algae but none of these bacteria were capable of forming microcysts, a feature which distinguishes them from the Myxococcus PCO2 isolate. Myxobacter has been a general name for any bacterium falling within two orders, Myxobacteriales and cytophagales. Using the criteria described by Stewart and Brown (1971) their isolates would be grouped as members of the Cytophaga genus by the 8th edition of Bergeys manual (Buchanan and Gibbons, 1974).

Daft and Stewart (1971) described four myxobacter that could lyse 40 strains of bluegreen algae. Again cell contact appeared to be necessary for lysis to occur. The authors suggested that one bacterium can initiate lysis of the algae. Although lysis took from 2 to 7 days photosynthesis was inhibited about 85% after 10 hours. Daft and Stewart (1971) indicate that these myxobacteria may be important in regulating algal development in nature.

The structural basis for algal lysis by the Myxobacterium CP1 was described by Daft and Stewart (1973). The primary ultrastructural effect was the dissolution of the L2 or mucopeptide layer in the cell wall of the bluegreen algae tested. Large intrathylakoidal spaces were seen to form; however, the membranes themselves seemed very resistant to myxobacter CP1 disruption. This pathology of the photosynthetic system is very similar to that described for bdellovibrio interaction with Phormidium luridum (Burnham and Sun, 1977). Daft and Stewart (1973) point out that the concentration of bacteria employed in these structural studies were far in excess of those encountered in the fold. Generally, a 1:1 dilution of the bacteria with algae were employed.

The physiologic conditions under which algal lysis by various myxobacteria occurred was reported by Daft et al. (1975). The bacteria were all strict aerobes. Lysis increased as the pO_2 was increased to 45%. Higher levels were

inhibitory. The pH optima for lysis was within the range of 7.0 to 9.0 for all strains of myxobacteria tested.

Lysis was not reported at 37C for strain CP1. Daft et al. 91975) suggest that optimum lysis in the field should be expected in the summer months in shallow water as the pH will also be quite suitable. These authors showed that in surveying 8 bodies of water in Scotland (5 lakes, 2 reservoirs and 1 sewage plant) there was always a direct statistical correlation between chlorophyll a concentration in the water and the abundance of these lytic bacteria. The number of myxobacteria per ml of lake water ranged from 4 to 400.

Myxobacterial Lytic Enzymes

The following reports show that the myxococci have significant lytic enzymes which could be important in carrying out the lysis of captured cyanobacterial prey cells.

In studying the myxobacter strain AL-1, Ensign and Wolfe (1966) described an enzyme possessing both proteolytic and cell wall lytic activity. These two functions were inseparable upon purification.

Hart and Zahler (1966) studied a lysin produced by M. xanthus FBa. Purification yielded two distinct enzymes, a lysozyme and a protease. The lysozyme was very effective in lysing cell walls of various microorganisms.

Further purification of M. xanthus FB bacteriolytic enzyme was described by Sudo and Dworkin (1972). By gel separation techniques an amidase, a glucosaminidase, two proteases with amidase activity and a peptidase active against cell wall peptides were isolated. These are all individually capable of bacteriolytic activity and collectively they appear to indicate why the Myxococcus and its related genera are such potent antimicrobial parasites.

Haska (1974) purified the peptidase produced by a related species, M. virescens and identified it to be a D-alanyl-N lysine endopeptidase, an enzyme that would cause the destruction of the L2 (mucopeptide) layer as observed by

Daft and Stewart (1973).

An alternative mechanism for the lysis of algal species could relate to the autolytic system that has been described for M. xanthus FB (Kottel and White, 1974). This enzyme system is induced during microcyst formation. The release of these enzymes which appear to result in the dissolution of cell walls could lyse walls of sensitive algal strains. Wireman and Dworkin (1977) further characterized this autolysis in terms of its sequence in the morphogenetic events leading to myxospore development. The formation of the myxospore appears to be dependent on the concentration of lysis products which may provide the rationale for the autolysis.

Myxococcus xanthus strains have been shown to also produce an antibiotic active against both Gram-positive and Gram-negative bacteria. The antibiotic appears to be bacteriocidal as Escherichia coli B cells when exposed to it for 60 min showed all lysis (Rosenberg et al., 1973). Vaks et al. (1974) characterized the antibiotic to be colorless and to be active only against growing cells.

Finally, it has been reported recently thas proteases are bound to the extracellulr slime found associated with M. virescens B2 (Gnosspelius, 1978). the author suggests that these enzymes could play an important role in denaturing protein components from microbial cells lysed by myxobacterial activities.

Additional background information can be found in the introduction in the manuscripts included in the Appendix section of this report.

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RESULTS

The following represent the major findings resulting from the research conducted for this contract B-086-OHIO. Detailed results can be found in the manuscripts included within the appendix.

1. Three myxococci,i.e., two strains of M. xanthus and one strain of M. fulvus, formed colonial aggregates and lysed cyanobacteria under autotrophic conditions.

2. Light microscopy showed evidence of swarming activity on the surface of all three myxococcal colonies with accompanying formation of fruiting structures.

3. Extended incubaton of mixed cultures showed the myxococci to be capable of long-term control (2 months) of the cyanobacterial population.

4. Predator-prey cycling occurred on average every 9 days of culture.

5. Serial transfer of the myxococci into healthy cultures of P. luridum showed the persistence of predatory activity without any subsequent exposure to heterotrophic nutrient.

6. Myxococci were shown to repeatedly multiply under predatory conditions to approximately 10^7 cells/ml.

7. Myxococci predatory inoculum levels could be as low as 50 cells per 100ml and effectively destroy a 10^7/ml population of P. luridum.

8. Predatory activity could be initiated with a pure inoculum of myxococcal spores of M. fulvus.

9. All three strains of myxococci have a wide spectrum of predatory activity against the cyanobacteria; however, not all strains or species are equally susceptible.

10. M. fulvus effectively entrapped cells of Chlorella vulgaris but was unable to lyse them.

11. Extended starvation of the myxococci by suspension in Difco Algae Broth for up to 60 days did not affect the cells (mixture of vegetative cells and spores) ability to form colonies and lyse populations of cyanobacteria.

12. Optimal temperature for predatory activity was 28-30C. Predation was greatly inhibited at 15C and 37C.

13. Predatory activity occurred effectively between pH 6.0 and 8.0.

14. A model of myxococcal predatory activity was designed and explained.

15. Myxococcal colonies form by entrapment of long lipopolysaccharide extrusions and myxococcal fimbriae.

16. A fimbriae-lacking mutant of M. fulvus was unable to lyse liquid cultures of P. luridum but did lyse the cyanobacteria when they were grown on agar lawns.

17. Copredation, i.e., the adding together of different microbial predators for cyanobacteria was tested.

18. Actinoplanaceae-myxococcal cultures showed successful joint colony formation and the survival of both predators in this configuration.

19. Copredatory colonies did lyse cyanobacterial cultures but not to the synergistic extent we had been looking for.

20. Some copredatory situations actually resulted in increased algal productivity.

21. Myxococcal predation was examined in dense stable multispecies algal microcosms.

22. Predation was measured in the first several days of interaction but its effect was lost after this period.

23. Inorganic carbon balances were followed during this myxococcal predation in microcosms.

24. Myxococcus fulvus was grown in two continuous flow systems, i.e., a) within a) 750 ml pot or b) upon glass beads organized in a 68 cm vertical column.

25. M. fulvus cells produced lytic enzymes in both systems and were able to entrap and lyse autotrophic P. luridum continuously.

PRESENT STATUS

Although the long-range goal addresed in this project has not been fully accomplished we have progressed positively toward it. Control over unialgal cultures has been achieved and our continuous flow culture experiments show the myxococci to be effective trapping agents of cyanobacteria in flowing systems. Because the data this research has generated is positive toward our goal we are continuing the research utilizing a multispecies host system. We plan to test the effect of culture complexity in order to predict the behavior of myxococci and natural ecosystems. The subsequent step will be to proceed to field testing.

Research grants for this work are being sought from the Ohio Water Resources Center, the Ohio Sea Grant Program and the National Science Foundation.

RECOMMENDATIONS RESULTING FROM THIS PROJECT

1. My data suggests that myxococi can be effective control agents for cyanobacteria as unispecies of these microortanisms were found to be entrapped and lysed by aqueous colonies of the myxococci.

2. Because of this finding I recommend that this microbial predator system be tested in increasingly complex environments in order to determine the limits of successful predation.

3. Once the behavior of the myxococci in multiple species planktonic environments is understood only then should field testing be conducted. There have been too many negative experiences from the uncontrolled introduction of exotic predators in specific environments to justify blind introduction of large amounts of these myxococci into an open system.

4. Continued research into the behavior of the myxococci in simple and multiple speciated planktonic environments should provide significant information regarding the behavior of indigenous myxococci in natural ecosystems. I strongly recommend the direction of the research described in this report to continue in order to provide this needed understanding. For example, why, if there are approximately 40 myxococci and other algal lytic bacteria present per ml of pond water, are these indigenous potential predators apparently unable to control cyanobacterial populations, particularly in eutrophic conditions. I believe answers to these questions will follow from pursuit of this research.

PERTINENT REFERENCES ORIGINATING FROM THIS GRANT PROGRAM

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APPENDIX REPORTS

1. Burnham, J. C. 1983. Structural perspectives on myxococcal predation.

2. Burnham, J. C. and P. C. Fraleigh. 1983. Predatory myxobacteria: lytic mechanisms and prospects as biological control agents for cyanobacteria (bluegreen algae).

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Structural Perspectives on Myxococcal Predation

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Microscopic studies of the predator-prey interaction between either Myxococcus xanthus or Myxococcus fulvus and the cyanobacterium, Phormidium luridum demonstrate that lysis of the cyanobacterium occurs within the core of the myxococcal colonies. The earliest interactions result in irregular microclumps of vegetative rod-shaped myxococci and cyanobacterial filaments forming in the planktonic environment. As these clumps mature, colonies 1 to 6 mm in diameter develop. Electron microscopy shows that long extrusions from the outer membrane of the myxococci are involved in both the formation of the initial clumps and of the mature colonial spherules. Thin sections of the lytic colony cores show captured cyanobacteria in various stages of enzymatic degradation. Both light and electon microscopy suggest strong similarity between the development of fruiting structures in colony forms growing on solid surfaces and in the planktonic environment. It appears that fruiting body formation with concommitant myxospore development results in lytic enzyme secretion by the myxococcal population. The structural evidence suggests that the encapsulation of these enzymes by the vegetative mass of the myxococcal colony allows sufficient enzyme density to be retained to result in prey lysis.

PREDATORY MYXOBACTERIA: LYTIC MECHANISMS AND PROSPECTS AS BIOLOGICAL CONTROL AGENTS FOR CYANOBACTERIA (BLUE-GREEN ALGAE)

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An invited paper presented at the Conference on North American Lake Management, Vancouver, British Columbia.

INTRODUCTION

Huffaker et al (1976) indicated that the premise of successful biological control was based on the fact that organisms have "natural enemies". These authors indicate that using these "natural enemies" may provide the best approach to developing a biological control agent. These control agents must possess several attributes if they are to be successful. These are: adaptability to physical conditions; searching (or trapping) capacity; multiplication; power of prey consumption; and survival during periods of low prey availability. We will present the myxobacteria as bacterial organisms which fit these requirements.

The relationships between bacteria and cyanobacteria involve symbiotic, commensal and antagonistic interactions. Although the most common relationships in nature are symbiotic (Echlin and Morris, 1965; Lange, 1971) naturally occurring antagonism has been described by Fitzgerald (1969). He demonstrated that bacteria-dominated sewage antagonized the cyanobacterium Microcystis aeruginosa but not the green alga Chlorella. Fallon and Brock, 1979, showed that an antagonistic bacterial population of 10^3 per ml were present in a Michigan lake and that these bacteria depended on the degraded products of cyanobacteria. Gunnison and Alexander (1975) described the peptidoglycan cell wall layer of the cyanobacteria as the "weak link" in their resistance to natural antagonistic bacteria. Our paper will amplify this by examining the development of information about the predatory myxobacters and describe in detail the mechanism of the myxococci in lysing captured cyanobacteria. Finally our recent research efforts in using (a) co-predatory colonies of myxococci and actinomycetes against aqueous cyanobacteria, and (b) stable microcosms as ecosystem testing systems for myxococcal biological control will be presented.

HISTORY

The bacteriolytic ability of myxobacteria has been recognized for a long time (Beebe, 1941) but it has only been since 1967 that their ability to lyse cyanobacteria has been described (Shilo, 1967). Earlier reviews are available of the subsequent papers which have appeared describing the myxobacterial lysis of cyanobacteria (Burnham, 1975; Burnham et al.,1981; Burnham, 1981; Stewart and Daft, 1977) so the details of many of these papers will be omitted here. Stewart and Brown (1969) showed that a Cytophaga could form plaques on lawns of both green and bluegreen algae. These authors described the algsl lysis as extracellular. Shilo (1970) showed that a myxobacter isolate (FP-1) could attach directly to the cell surface of the cyanobacteria and subsequently lyse the host cell. Although detection of secreted lytic enzymes was unsuccessful, the fact that algal lysis was prevented when the combined cultures were shaken suggests that an exoenzyme is involved and that the agitation removed the necessary enzyme concentration from the microenvironment set up by the attached cells.

A similar problem was encountered by Daft and Stewart (1971; 1973; 1975) in using several Lysobactersp isolates to lyse cyanobacteria. They found agitation interfered with lyses and that high densities of the lytic bacteria were necessary for successful aqueous predation. This was a result which conflicted somewhat with their survey results of Scottish locks and reservoirs. These bodies of water all contained lytic myxobacteria in concentrations ranging from 1 to more than 100/ml with a mean of 44/ml in seven different habitats. A direct myxobacterial relationship with prey populations was proven by these authors by showing that the population of myxobacteria was in direct proportion to the chlorophyll a levels in these waters. What they did not show and what we do not know as yet is whether these predatory bacteria have any effect on the ecosystems present in these waters.

Daft and Stewart (1973) demonstrated ultrastructurally that it was the peptidoglycan layer that was theprimary target of the myxobacterial enzymes providing evidence for the later theory of Gunnison and Alexander (1975).

It is possible, in analyzing Daft and Stewart's data, that aqueous colonies of myxobacteria did form, as their enumeration methodology could have underestimated the predatory population level. The idea that colonies of predatory bacteria might be involved in aqueous lysis of cyanobacteria is supported by the results described by Burnham et al (1981). Myxococcus xanthus PCO2 has been shown to form semispherical colonies which entrapped and lysed populations of the cyanobacterium Phormidium. The details of this lytic mechanism will be described in the next section of this paper; however, it should be mentioned here that study of this lytic mechanism has provided further evidence that successful lysis in aqueous ecosystems depends upon the establishment of a protected environment. This then allows the cell wall lytic enzymes to accumulate to an effective concentration. This mechanism is very analyogous to that utilized by the bdellovibrios, a predatory group of bacteria that can penetrate into and lyse various Gram-negative bacteria. Rittenberg and Thomashow (1980) described the bdellovibrio predator as forming a "cozy environment" within the confines of the cell wall of the host cell. Here suitable enzyme concentrations can be maintained for the orderly transfer of nutrient from the prey cytoplasm to the predator. The myxococci apparently do essentially the same thing in providing a "cozy" or protected environment for enzymatic activity, but on a multicellular basis.

MYXOCOCCAL PREDATION

In this section we will review the major characters of this myxococcal lytic system. Much of this research has been previously reported (Burnham et al, 1979; 1980a; 1980b; 1981; 1983).

In combined culture, with a sensitive strain of cyanobacteria (9 species have been found susceptible, Burnham et al, 1983), the myxococci will form semispherical colonies in which the prey cyanobacteria became concentrated in the middle of the colony (Fig. 1) surrounded by a massive multicellular border of myxococcal vegetative cells (Figs. 1, 2).

This ability to form a core provides a protected environment in which the lytic enzymes produced by the myxococci can digest the entrapped prey. The enzymes produced by the myxococci can digest the entrapped prey. The enzymes for this lytic process appear to relate to the system of autolysis and spore production which characterize this Myxococcus genus (Kottel and White, 1974; Wireman and Dworkin, 1977).

Figure 3 shows the fruiting structures, comprised of swarming vegetative cells and myxospores, that develop on the surface of the Myxococcus fulvus BG02 strain when grown in agitated cultures of Phormidium luridum. Various enzymes including two proteases with amidase activity, a peptidase, an amidase and a glucosaminidase have been isolated (Sudo and Dworkin, 1972). It appears that the formation of myxospores depends upon a cell-free concentration of cytoplasmic constituents from lysed myxococci. The cellulytic enzymes are needed for this autolysis. Because the enzymes produced by individual myxococci either diffuse out of the colony or diffuse to the core of the colony, it is possible to propose that this colonial core becomes an enzyme sink gradually increasing in enzyme concentration. Enzymatic digestion of the prey predominantly occurs in this core with subsequent uptake of this liberated nutrient by the myxococci in the periphery of the colony.

The entrapment mechanism appears related to the fimbriae (Dobson and McCurey, 1979) and the lipopolysaccharide extrusions (Burnham et al, 1981) present on the surface of the myxococcal vegetative cells (Fig. 4). These appear to bind (or entangle) the cyanobacteria onto the colony surface. The entrapped cyanobacteria are gradully moved to the core of the colony via the swarming (due to gliding motility) of the cyanobacteria on the colony surface. The entire process of entrapment, translocation, lysis and nutrient transfer is diagrammed in Fig. 5. It is important to realize that this takes place under autotrophic conditions. Therefore, all nutrient for the survival and development of the myxococci must originate with the cyanobacterium. That this process is efficient is demonstrated by the curve in Fig. 6 (from Burnham et al, 1983) which shows the sequential lysis of a fresh culture of the cyanobacterium P. luridum about

every 7 days for a period of over two months. The myxococci were serially transferred at a volume ratio of 1%. We found inoculum levels could be very low. If 500 individual myxococci were present per ml, lysis of $3x10^7$ P. luridum/ml population occurred in about 7 days. In other words, it appears that the myxococci required a lag period to develop the colony structure necessary for large scale lysis of the cyanobacterial population. It is possible that early growth and development of colonies from such low inocula is in part due to the myxococci utilizing the secretions of viable cyanobacteria (Daft et al., 1975). Ward and Moyer (1966) showed that bacterial populations could increase by utilizing algal secretions when they demonstrated that the number of bacteria in an autotrophic mixed culture multiplied relative to the amount of algal growth.

CO-PREDATION

In order to increase the potential of the myxococcal predatory system, the concept of copredation was developed using an actinomycete and the Myxococcus fulvus BG02 together in lytic colonies. Much of this work was done with Dr. Melvin Daft at the Department of Biological Sciences, University of Dundee, Dundee, Scotland. Figure 7 is a micrograph of one such colony formed by an actinomycete obtained from Dr. Daft and the M. fulvus BG02. These copredatory colonies were formed by placing equal numbers of single cells in a dilute medium (Difco Algae Broth plus 0.2% Bacto tryptone) and shaking the mixture for 24-48 hrs. The colonies that resulted often appeared as in Fig. 7 with the actinomycete predominantly occupying the core of the colony and the Myxococcus predominantly present in the periphery. Light microscopy of these colonies shows (Fig. 8) that the surface protrusion from this colony contained intertwined vegetative rod forms of both predatory species. When these colonies were placed in cultures of P. luridum, lysis of the cyanobacteria occurred. Figure 9 shows the chlorophyll a level remaining in an agitated aqueous P. luridum culture after 9 days

of interaction with both single and copredatory colonies. The copredatory systems using either of the actinomycete strains C9 or N6 showed some increase in lytic activity but not to the extent we had hoped for. Part of the reason appeared to be due to a less efficient trapping and translocation mechanism. We suggest this was because the actinomycete interferred with the swarming activities of the Myxococcus and actually filled the core of the colonies (Fig. 7) reducing the space available for cyanobacterial digestion. Phase contrast microscopy of lysing P. luridum indicated that in these copredatory colonies most of the cyanobacterial lysis appeared to be taking place among the layers of the periphery of the colony. When other species of cyanobacteria were exposed to thse copredatory colonies, specifically Anabaena cylindrica or Nostoc muscorum, the amount of chlorophyll a present per ml in the system actually increased over time instead of decreasing as was expected. This result was confirmed by following nitrogen fixing activity of the cyanobacteria; i.e., the copredatory systems became more productive than the controls (axenic cyanobacteria in autotrophic medium). It appears that this effect is due partly to the interference by the actinomycete as discussed above, and partly to the increased nutrient available from cyanobacterial lysis that stimulated the growth of the remaining cyanobacteria--in other words, the growth rate of the prey species in the copredatory system outstripped the killing activity of the predators. It must be remembered that this system utilized equal numbers of both predators; we hope it will be possible to improve on this system by changing the ratio of the predatory partners and eventually achieve synergistic predation of the cyanobacterial prey.

MYXOCOCCAL PREDATION IN ALGAL MICROCOSMS

Studies of stable algal microcosms (Fraleigh and Dibert, 1980; Cooke, 1967) have provided an understanding of the effect of nitrogen, phosphorus and inorganic carbon concentrations on algal standing crop. These data should provide an excellent basis for both experimental design and evaluation of myxococcal effects. We believe these microcosms to be excellent complex systems in which to test the lytic ability of the myxococci. This is consistent with the earlier recommendations (Burnham, 1981) that

evaluation of myxococcal biological control be carried out in increasingly complex systems before large scale introduction into pond or lake ecosystems. We would like to report some of our preliminary investigations using these systems as they provide some basis for predicting the usefulness of myxococcal biological control.

Stable algal microcosms were produced in a modification of the salts medium of Taub and Dollar, 1964 by the method described by Fraleigh and Dibert, 1980. These were cultured from the same stocks which had been used in the inorganic carbon studies of Fraleigh and Dibert, 1980. We identified both green algae and cyanobacteria present in high concentrations. The dominant genera in our microcosms appeared to be Microcystis, Phormidium, Oscillatoria, Hapalosiphon, Chroococcus and Dispora. Also present were ostracods and an amphiod. Figure 10 shows a control stable microcosm with the very dense algal community primarily residing in the bottom of the water column. The test microcosms were not shaken and were not aerated during exposure to the predator. Several six-month old microcosms were pooled, distributed (750 ml) into 1 liter Erlenmeyer flasks and experimental cultures were inoculated with high levels of myxococci (10^8/ml and 10^7/ml) to maximize any effect on the algal standing crops.

While inoculation with the low dose of myxococci did not appear to have a significant effect in the microcosm, the high dose did. In both the control and low-myxococci microcosms there appeared to be a maintenance of a diversity of algal species and changes in chlorophyll a concentration were similar (Fig. 11). In contrast, chlorophyll a concentrations in the high-myxococci microcosms increased to about twice those in the low-myxococci and control microcosms. This was apparently related to an increase in the densities of Microcystis. A sample from one of the high-myxococci microcosms is shown in Fig. 12. This illustrates the high standing crop that resulted as well as one of the major obstables for myxococcal predation, i.e., the colonial form of growth of Microcystis. Thus, what appears to have happened is that the standing crop of a resistant algal species increased in the high-myxococci microcosms.

These changes in the high-myxococcal microcosms appear to have occurred as a consequence of the interaction of two factors: selective predation and nutrient recycling. In other ecosystems, selective predation has been found to result in an increase in species diversity and an increase in the relative abundance of non-preyed upon species at the prey trophic level (Paine, 1969 - the intertidal; Brooks and Dodson, 1965 - lakes; Hall, Cooper & Werner, 1970 - ponds). Consequently, the increase in relative abundance of Microcystis in the high-myxococcal microcosms was probably, in part, a consequence of selective predation by the myxococci preferred prey. However, recycling of nutrients as a consequence of myxococcal predation was probably also important. This factor may have been responsible for the increase in standing crops of chlorophyll a and of the Microcystis.

Shortly after inoculation of myxococci in the high-myxococcal microcosms pH decreased, the concentration of inorganic carbon increased and the percent saturation of CO_2 increased (Figs 13, 14). These appear to have been due to CO_2 released from myxococcal respiration and respiratory activities of organisms feeding on organic matter supplied as a result of myxococcal predation. These changes did not occur in the control (Fig. 15) or low-myxococcal microcosms. However, they did occur in the cultures of myxococci with Phormidium and of myxococci alone. From the large effect in the cultures of myxococci alone it seems reasonable to conclude that much of the change in inorganic carbon and CO_2 percent saturation in high-myxococcal microcosms and in the myxococci and Phormidium cultures was due to myxococcal respiration. Furthermore, because Fraleigh and Dibert (1980) have found that inorganic carbon can be limiting in these microcosms, the respiratory activity of the myxococci in increasing the inorganic concentration in the environment probably stimulated algal growth and contributed to the increase in the standing crops of chlorophyll a and Microcystis. This may also have occurred in the co-predation experiment with Anabaena and Nostoc described earlier and may have contributed to the increase in chlorophyll a in these experiments. In addition, recycling of other nutrients, as a consequence of myxococcal predation and breakdown of organic matter in prey algal species, and the stimulated

metabolism of saprophage populations, also probably contributed to the increase in chlorophyll a standing crop in the high-myxococcal microcosms. By day 6, a holotrichous ciliate protozoan had become abundant in the high-myxococci microcosms. These were probably feeding on a combination of bacteria and detritus from myxococcal predation and likely contributed to nutrient recycling back to primary producers.

Thus, what appears to have occurred in the high-myxococcal microcosms is the following sequence of events: Upon inoculation, the myxococci began preying on sensitive algal species. Myxococcal predation then provided soluble organic matter for saprophagous bacteria and these bacteria, myxococi, and particulate detritus from myxococcal predation provided a food resource fo eukaryotic particulate saprophages. The metabolism of all of these yielded a high rate of CO_2 production, probably a large release of other nutrients, and heterotrophic conditions in the microcosms (as evidenced by the free CO_2 concentration being supersaturated at both lights-on and lights-off between days 1 and 5). This then apparently stimulated growth on non-susceptible species (in this case Microcystis) resulting in an increase in the standing crop of chlorophyll a and subsequently a return to autotrophic conditions in the microcosms. That recovery occurred is indicated by the finding that free CO_2 became undersaturated at both lights-on and lights-off after day 6, a situation that would occur only if the rate of photosynthesis exceeded the rate of respiration over a 24-hour period. Consistent with this were conditions in the myxococci---Phormidium and myxococci alone cultures. In these, where resistant primary producers were absent, the CO_2 concentration remained supersaturated.

This scenario is probably similar to that which would occur in a natural ecosystem. Encouraging about these results, which are preliminary at best, is that the myxococcal predator can apparently cause a directional change in the species composition of the primary producer trophic level. Thus, while this system as utilized apparently does not have the capacity for broad spectrum control of algae it may be adaptable to situations where problems are due to one or several species. In addition by redesigning the predatory system, for example by a) incorporation of

copredatory colonies; b) utilizing preformed myxococcal predatory colonies; and c) introducing agitation, we believe that more active predation can be achieved over a larger time period. Not only may control be possible but conditions may be created favoring more desirable algal species and the energy resources tied up in the problem species may become available in the food chain leading to fish.

SUMMARY

To control problem growths of primary producers in lakes and ponds. especially blooms of blue-green algae and high densities ofmacrophytes, a diversity of methods have been proposed and are used. However, absent in this repertoire are methods of biological control analogous to those that have been successful in terrestrial ecosystems. Presented here is a discussion of studies which suggest that myxobacterial predation may be useful in biological control of blue-green algae in aquatic ecosystems. In cultures of Phormidium luridum inoculated with the predator Myxococcus fulvus BGOw strain, control of the blue-green algae was found and mechanisms of the predatory interaction have been described. Studies of co-predation by BGO2 and two strains of actinomycetes suggest some interference and a variable enhancement of BGO2 effectiveness. In fact, we were surprised to find that in some cultures with less instance, co-predation resulted in an increase in algal biomass (chlorophyll a). Lastly, preliminary work with microcosms containing a diversity of algae suggests that selective predation by Myxococcus occurred and densities of less sensitive algal species increased. In the microcosms, predation by the Myxococcus also resulted in a drop in pH, and an apparent increase in density of protozoa resulting in the microcosm becoming temporarily heterotrophic. These results are encouraging in suggesting that biological control of certain lake problems may be possible. Specifically where problems exist due to an excessive abundance of one or several blue-green algal species, introduction of a myxococcal predator may result in control of these species and creation of environmental conditions (esp. a locatized lowering of the pH) favoring more desirable algal species and may also increase energy flow to fish. The latter would occur if myxococcal predation made energy stored in otherwise unusable blue-green algae more available to heterotrophs in food chains leading to fish. Needed at this time are further studies with microcosms as a prelude to field tests. The objective of these would be to focus on and identify the behavior of the pedator in a community oforganisms. These results would then be useful in designing efficient studies in natural ecosystems to determine effect of added predators and determining if natural populations of predatory bacteria play a role in shaping ecosystem communities.

LEGEND FOR FIGURES

PLATE I

Figure 1. Phase contrast micrographs of an M. xanthus PC02 colony containing the cyanobacterium Phormidium luridum in the darker core. The lighter periphery of the colony is composed of swarming masses of myxococcal vegetative rod-shaped cells. 200X.

Figure 2. Bright field micrograph of a section from a parafin embedded axenically grown colony of M. xanthus PC02, The mass of myxococcal vegetative rod-shaped cells can be seen enveloping the relatively hollow core. 325X.

Figure 3. Light micrograph of the fruiting structures (arrows) that have formed on the surface of a predatory colony of M. fulvus BG02 and P. luridum - 150X.

Figure 4. This scanning electron micrograph shows the rod-like M. xanthus PC02 cells joined together by small protrusions from the myxococcal cell wall. Transmission electron microscopy has confirmed that these are composed of lipopolysaccharide. The entanglement of the fibers holds the sphere into shape and suggests a role in the entrapment of the cyanobacteria. 7015X. (This figure is reprinted from Burnham, 1981).

Figure 5. Diagrammatic model of myxococcal predation of filamentous cyanobacteria in eutrophic waters (ew). The cyanobacteria (c) are represented to grow autotrophically utilizing light (hv) and inorganic salts (i). These filaments are captured by the colony using the extrusions (f) from the myxococcal cell walls. Translocation from the surface to the colony core occurs via swarming activity of the myxococcal vegetative cells (m). During their life cycle in the colony periphery the myxococci produce cellulytic enzymes (e) during the process of spore formation (myxocysts, mc). These enzymes lyse many of the rod-shaped myxococcal cells (lm). The enzymes diffuse either out to the aqueous environment or into the colony core where they accumulate. When the cyanobacteria enter the core they are digested

by these enzymes releasing nutrient (n) which is subsequently absorbed by the growing myxococci (m) in the colonial periphery.

Figure 6. Effect on P. luridum density of serial 1% transfers into mature P. luridum cultures (between 10^5-10^7 cells/ml). The initial culture contained approximately 10^8 P. luridum/ml and 10^5 M. fulvus/ml. Vertical dashed lines represent transfer points.

LEGEND FOR FIGURES

PLATE II

Figure 7. Bright field micrograph of a copredatory colony comprised of *M. fulvus* BG02 (located predominantly in lighter appearing periphery) and a cyanobacterial lytic actinomycete (located predominantly in the darker core region). 225X.

Figure 8. Phase contrast micrograph of the surface of the copredatory colony seen in Fig. 7. Intertwined cells of the actinomycete (a) can be seen along with the vegetative rod-shaped myxococci (m) forming a large pointed colonial protrusion. 1000X.

Figure 9. Bar graph illustrating the reduction in *P. luridum* chlorophyll *a* in 9-day old cultures due to the predatory activities of an *M. fulvus* BG02 alone (P + BG02); *M. fulvus* BG02 within copredatory colonies with each of two lytic actinomycetes (P + BG02 + C9 and P + BG02 + N6); and each actinomycete alone (P + C9 and P + N6). The *P. luridum* contained 10^7 cells/ml while the actinomycete predatory cultures were inoculated with 1 ml from 5-day axenic cultures in a yeast extract-glucose medium. Both the actinomycete and *M. fulvus* colonies were fragmented in a tissue grinder and mixed in Difco Algae Broth to allow formation of the combined cultures prior to their being introduced into the cyanobacterial prey cultures.

Figure 10. A stable algal microcosm (6-month old) showing the dense growth of the dominant cyanobacterial and green algal species (see text for description).

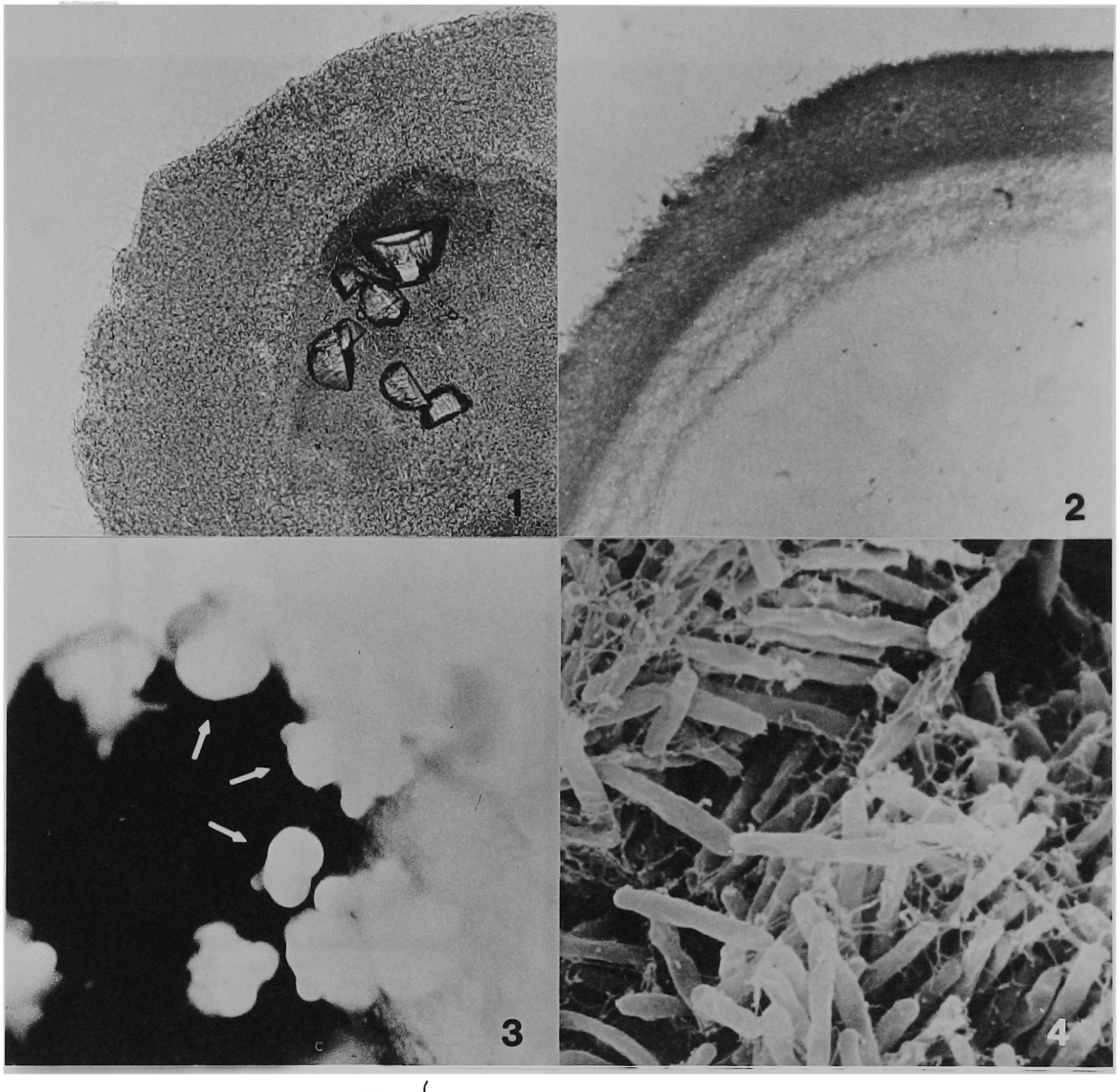
Figure 11. Changes in chlorophyll a in 5 ml acetone extracts of 10 ml samples of the benthic communities of control, low-myxococcal, and high-myxococcal microcosms. For each treatment there were 2 replicate microcosms. Time is in days after inocualation with myxococci. Chlorophyll a was determined by the acetone extract method and calculated using the trichromatic formula of Strickland and Parsons (1965).

Figure 12. Photomicrograph of the community in a microcosm 10 days after inoculation with a high dose of myxococci. The smallest separation on the micrometer scale is 2 um.

Figure 13. Changes in pH in control low-myxococcal, and high-myxococcal microcosms. Cultures were grown under a 16/8 hour light/dark cycle. Time is in days after inoculation with myxococci. S and R denote the beginning of the dark and light periods, respectively. For each treatment there were two replicate microcosms.

Figure 14. Changes in inorganic carbon concentration (from alkalinity titrations) and percent saturation of CO_2 (calculated from bicarbonate concentrations and pH) in experimental cultures - microcosms with high myxococci, microcosms with low myxococci and Phormidium cultures with high myxococci. Cultures were grown under a 16/8 hour light/dark cycle. Time is in days after inoculation with myxococci. S and R denote the beginning of the dark and light periods, respectively. For each treatment there were two replicate microcosms.

Figure 15. Changes in inorganic carbon concentrations (from alkalinity titrations) and percent saturation of CO_2 (calculated from bicarbonate concentrations and pH) in control cultures - microcosm without myxococci and myxococci alone (high dose).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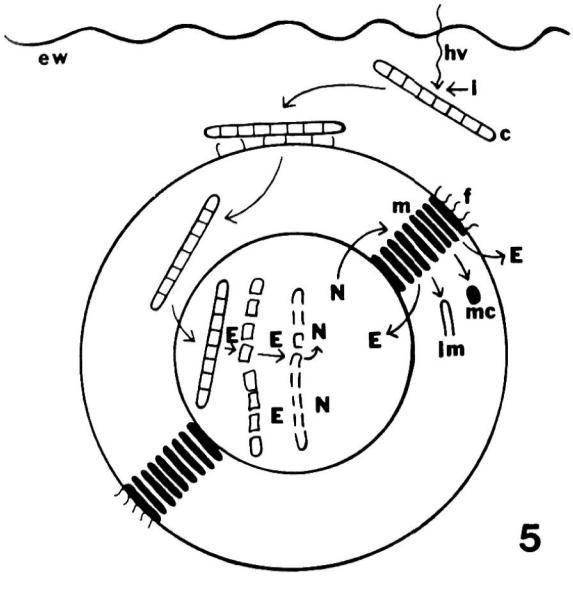
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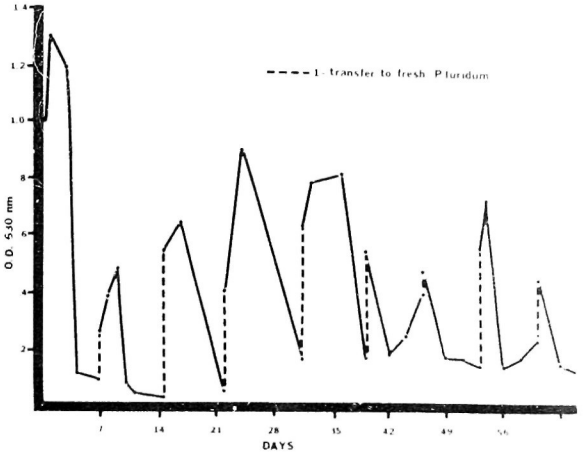
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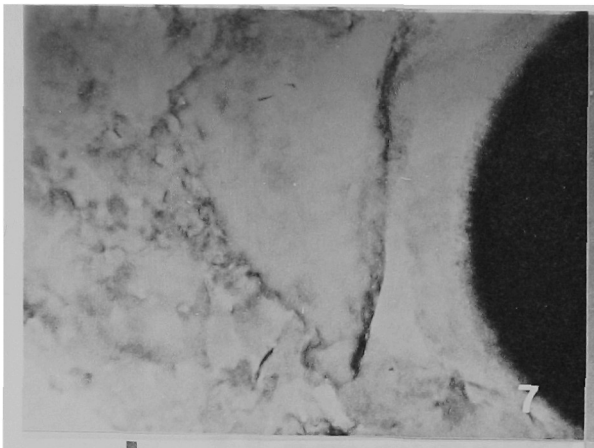

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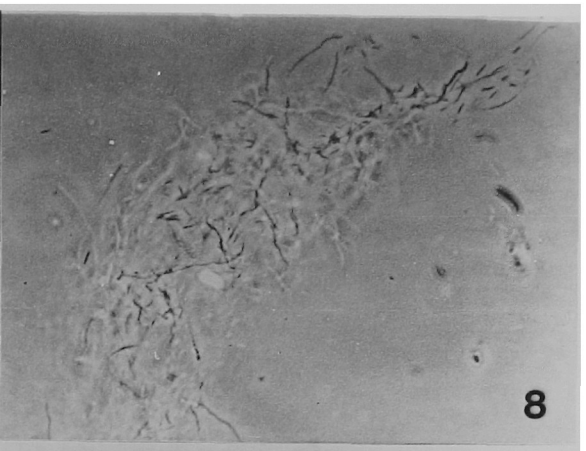

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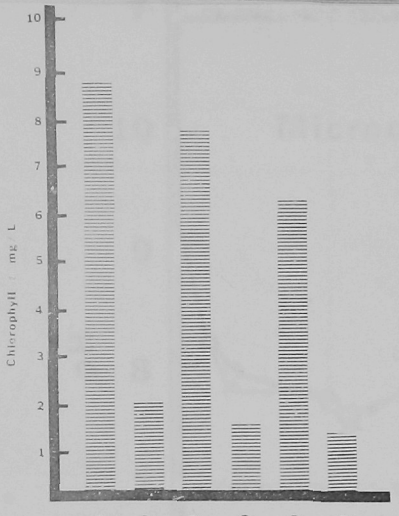
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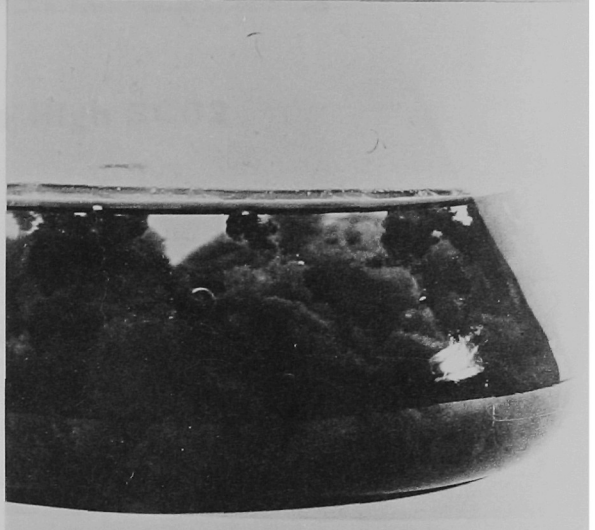

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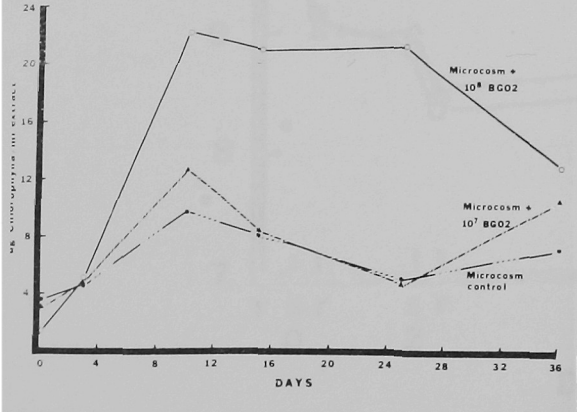
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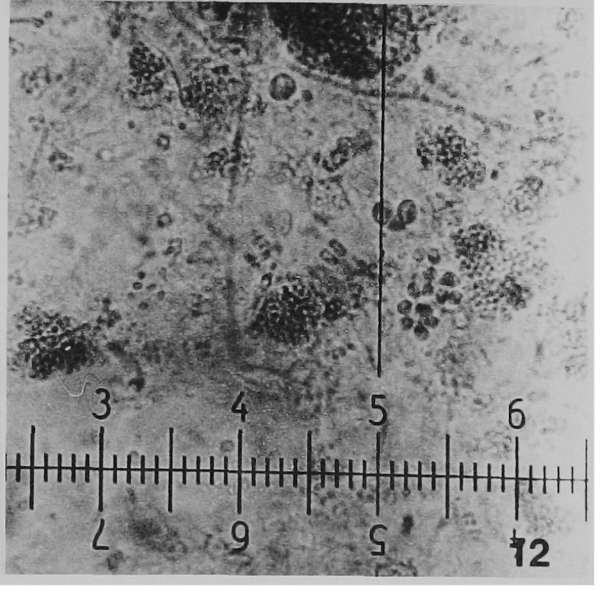
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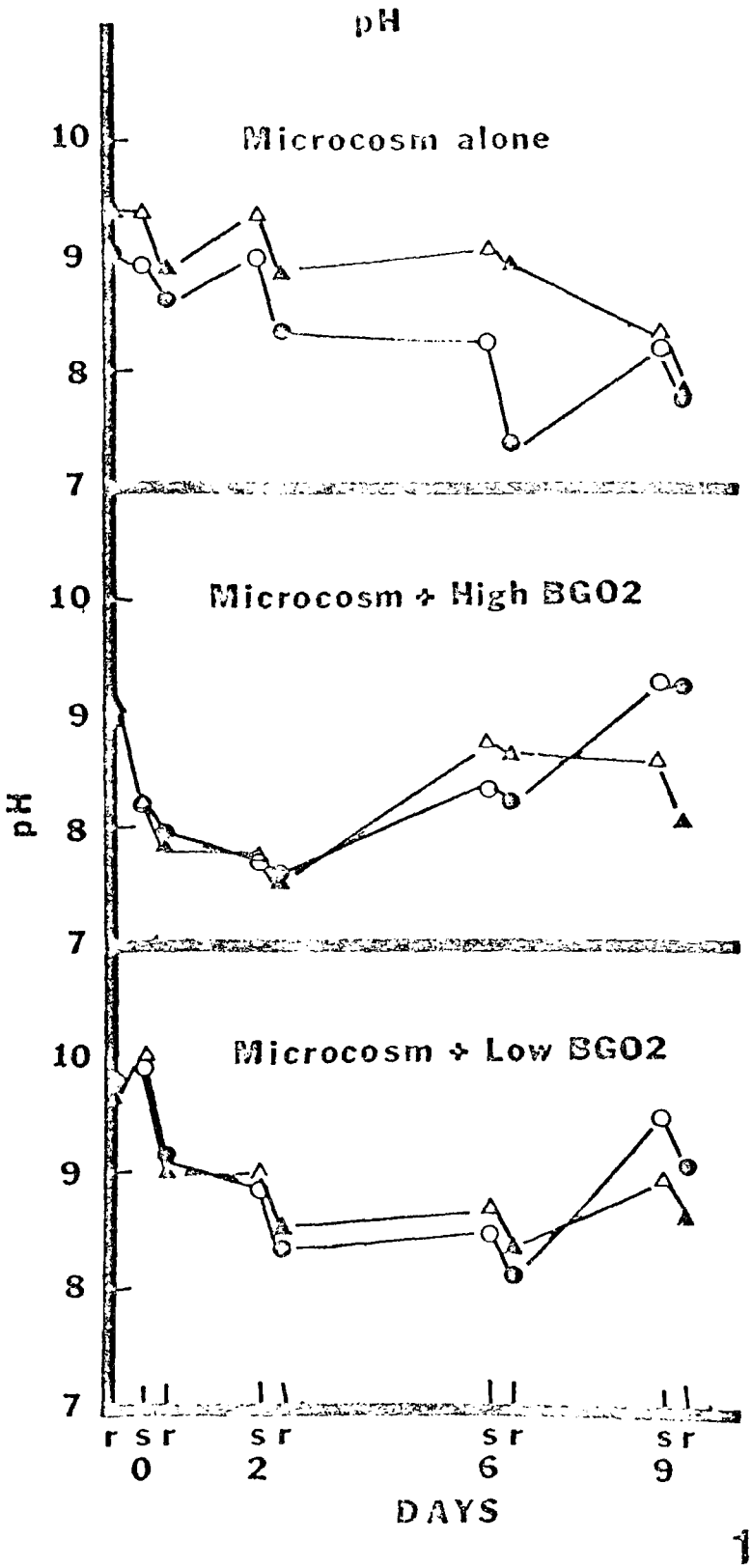
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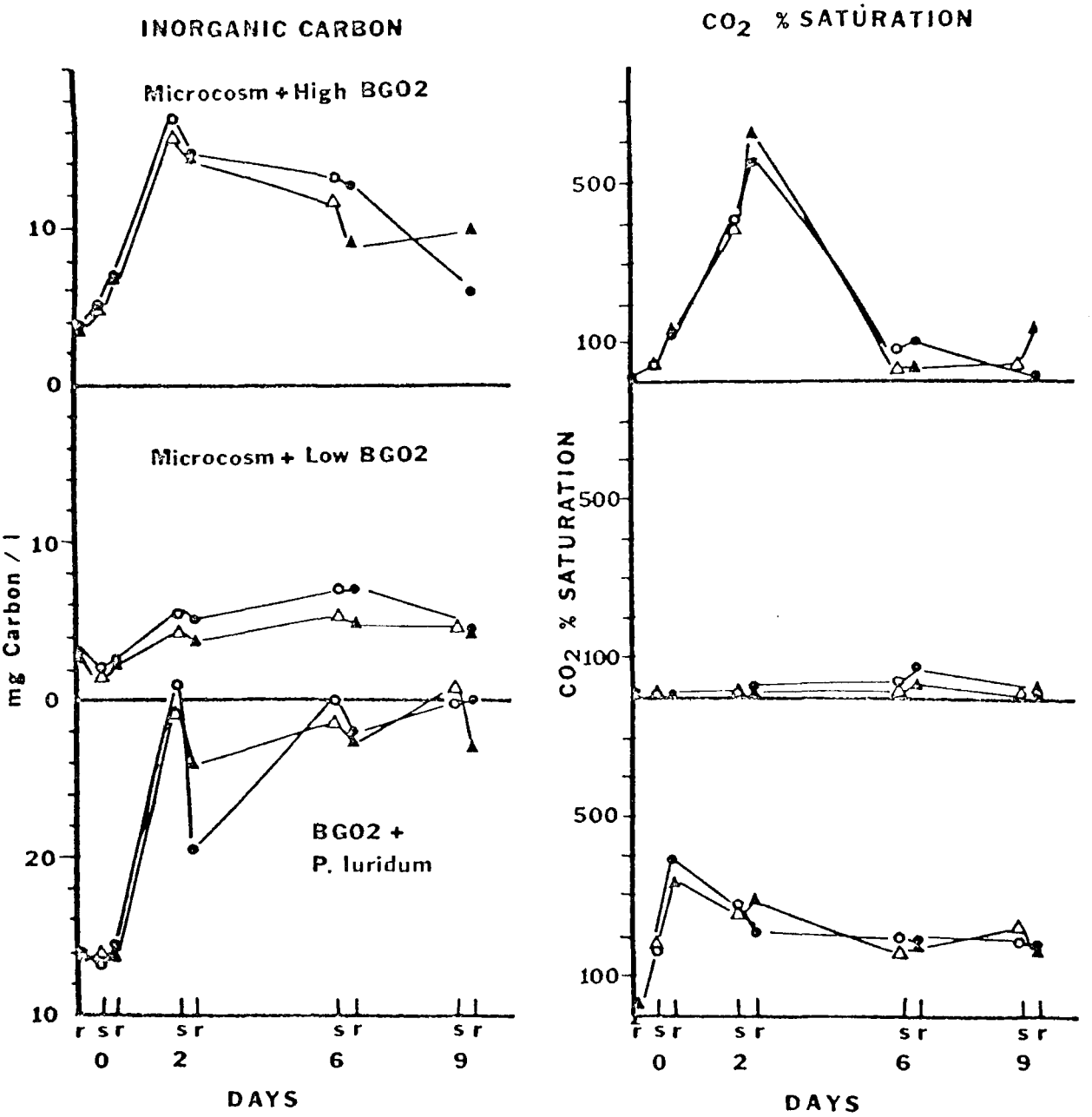
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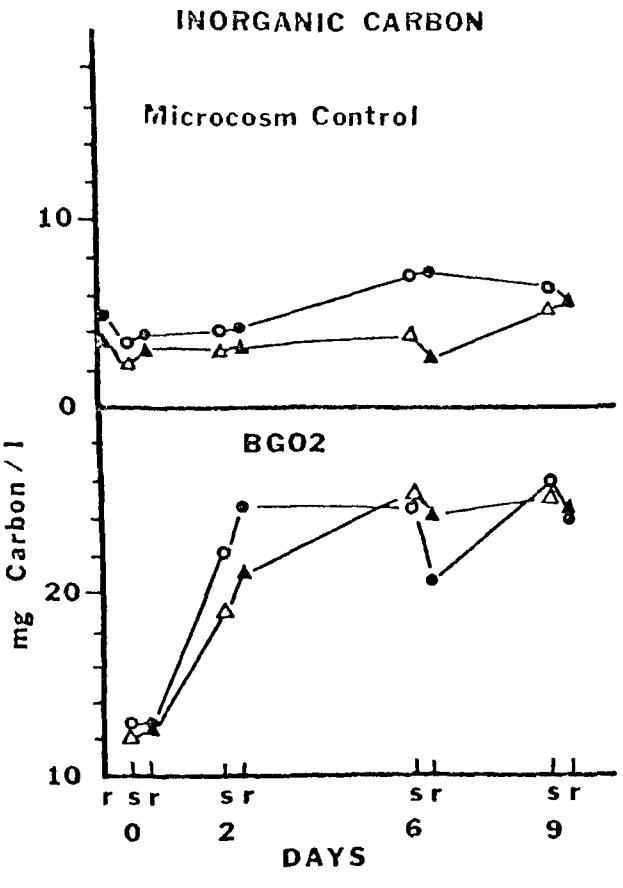
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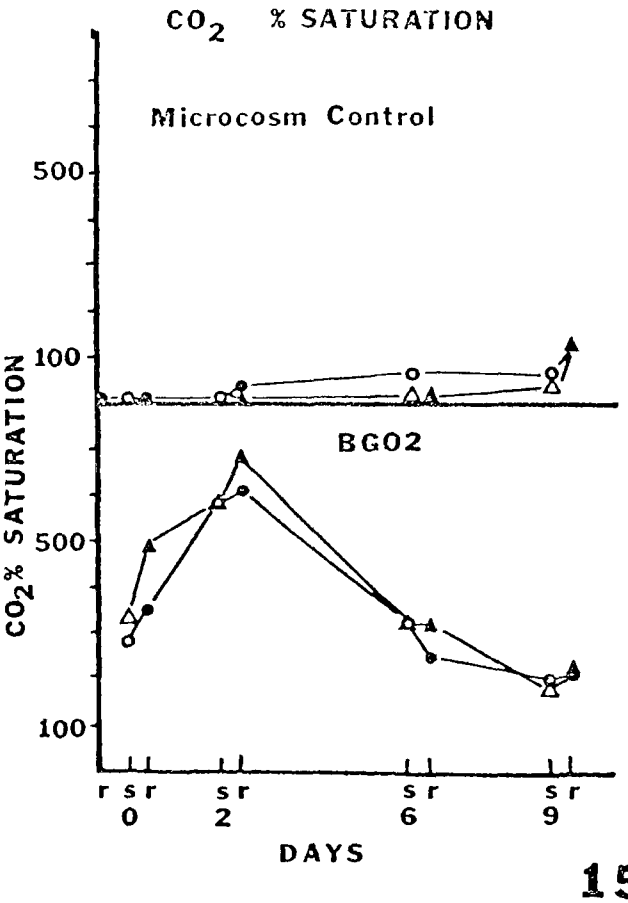
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Myxococcal Predation of the Cyanobacterium *Phormidium luridum* in Aqueous Environments

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Abstract. Two strains of Myxococcus xanthus, and a strain of Myxococcus fulvus were compared with respect to their ability to entrap and lyse trichomes of the cyanobacterium Phormidium luridum var. olivaceae. All of these isolates form colonial aggregates and spherules in either axenic culture with a tryptone-salts medium or in a mixed culture with viable cyanobacterial cells as the sole source of nutrients. Light microscopy showed evidence of swarming activity on the surface of all three myxococci with the accompanying formation of fruiting structures. Extended incubation of mixed cultures showed the myxococci to be capable of long-term control of the cyanobacterial population with predator-prey population cycling occurring on average every 9 days. Serial transfer of mixed cultures into either fresh autotrophic medium or cyanobacterial cultures of 10^7 per ml showed the persistence of predatory activity. Myxococcal densities were shown to return repeatedly to initial virulent levels. Predator inoculum levels could be reduced to 50 cells per 100 ml in a cyanobacterial culture of 10^7 per ml. These in vitro data enhance the potential of the myxococcus predatory colony as a biological control agent for in situ cyanobacteria.

Key words: Biological control - colony - cyanobacteria - entrapment - lysis - Myxococcus - Phormidium - predatory - prey - spherule.

In our first paper describing the relationship between spherule-forming myxococci and cyanobacteria in aqueous mixed cultures, the early stages of this predatory interaction were emphasized (Burnham et al., 1981). Microscopy showed that the myxococci formed colonial spherules in which the peripherally-located myxococci were able to concentrate the cyanobacteria within the core of the spherules and cause their lysis. Because the interaction was carried out under autotrophic conditions, all nutrients for myxococcal growth and development must have originated from the prey cyanobacteria.

Although the myxococci were shown to be effective over nearly three weeks no experiments had been conducted to measure predatory ability under population levels similar to those that have been described in nature (Daft et al., 1975).

Long-term relationships between bacteria and cyanobacteria are not uncommon. These appear to be predominantly of the commensal or symbiotic type (Lange, 1971; Echlin and Morris, 1965) such as that illustrated in the micrographs by Pearl (1976) showing the surface colonization of nitrogen fixing heterocysts of Anabaena by bacteria. Such relationships ahve been reported for the myxococci. Nolte (1957) showed that when either of two species of Anabaena were present as sole nutrient sources in mixed cultures with any of three species of Myxococcus, including M. fulvus, the myxococci were able to grow and form fruiting structures without lysing the cyanobacteria. That antagonism does exist in nature between bacteria and cyanobacteria has been demonstrated by Fitzgerald (1969) by showing that bacteria-dominated sewage would permit the growth of the green alga, Chlorella, but not the cyanobacterium Microcystis aeruginosa. Gunnison and Alexander (1975) examined the degradation of algae by bacteria and pointed to the peptidoglycan component of the cyanobacterium's cell wall as their "weak link" against antagonistic bacteria. Fallon and Brock (1979) in describing

the decomposition of cyanobacteria in a lake in Wisconsin showed that an antagonistic bacterial population of 10^3 cells per ml essentially depended upon the products of that degradation for their nutritional needs. Daft et al. showed that 44 lytic bacteria per ml effective against cyanobacteria could be isolated in Scottish waters. The significance of this relationship between these indigenous lytic cyanobacteria and the planktonic bacteria is questionable because of the finding by Daft et al. (1975) that 10^6/ml of one of these lytic strains were necessary to cause cyanobacterial lysis.

Although many details of cyanobacterial lysis by bacteria have been described particularly with reference to predatory strains (Shilo, 1970; Daft and Stewart, 1971; Stewart and Brown, 1971; and Stewart and Daft, 1977) resembling Lysobacter sp. (Christensen and Cook, 1978), none of these reports show the results of long term and serial transfer or low inocula experiments. This second paper describing the interaction between myxococcal strains and cyanobacteria in aqueous culture examines this long-term predatory and survival capability of the myxococci.

MATERIALS AND METHODS

Isolation and Identification

The myxococci used in this study were all isolated from grab samples obtained from roadside ditches draining agricultural fields in Northwest Ohio. Strains BG02 and BG03 were isolated in Bowling Green, Ohio while strain PC02 was isolated in Port Clinton, Ohio. Isolation was as described previously (Burnham et al., 1981) utilizing autotrophically grown lawns of the cyanobacterium Phormidium luridum. The characteristics of the PC02 strain, identified as a Myxococcus xanthus, have been described (Burnham et al., 1981). The BG02 and BG03 strains were identified using the criteria of Zahler and McCurdy (1974).

Organisms

The host cyanobacterium utilized in this study, Phormidium luridum var.

olivaceae (No. 426) was obtained from the University of Texas Culture Collection of Algae and is maintained bacteria-free on Difco Algae Agar or in Difco Algae Broth (AB) as described earlier (Burnham et al., 1976). The myxococci were routinely maintained on ABT medium (Difco Algae Broth containing 0.2% Bacto Tryptone) or on P. luridum lawns on Difco Algae Agar (Burnham et al., 1981).

Mixed Cultures. Routinely only the myxococci organized into colonial spherules were transferred directly from ABT cultures (1% v/v) and placed into 4- to 7-day-old autotrophically-grown P. luridum broth cultures. Various experiments were employed which necessitated the removal of all heterotrophic nutrients as well as cell quantitation. For these, the myxococcal spherules were removed by pipette from their ABT medium and washed twice with 5 volumes of algae broth. These spherules were then placed into a glass tissue grinder (Wheaton) and plunged over 50 times to disaggregate the vegetative cells comprising the spherule. These cell suspensions were diluted with AB medium to concentrations of approximately 10^6 bacteria per ml, as determined by microscope countig with a Petroff-Hausser chamber, and used for inoculations into aqueous P. luridum cultures. These inocula were immediately plated on ABT agar (Burnham, et al., 1981) for subsequent more accurate quantitation.

For long-term experiments presented in this paper, the initial and subsequent inocula upon serial transfer was 5% (v/v). The flasks were maintained at 25°C and, unless otherwise specified, in 16h light (3,200 lx) and 8h dark. The 500 ml Erlenmeyer side arm flasks were rotary shaken at 100 RPM.

Microscopy. Light microscopy was carried out with a Zeiss Axiomat Microscope using quartz halogen illumination. For scanning electron microscopy (SEM), all cells and spherules were directly fixed in 4% glutaraldehyde in 0.1M KH_2PO_4 at pH 7.2 for 12h. Small spherules were filtered onto Nucleopore membranes prior to dehydration. All specimens were

dehydrated and critical point dried as previously described (Burnham et al., 1981). Large specimens were coated with gold palladium in a Polaron SEM Coating Unit E5100 and examined in a Cambridge 180 SEM.

RESULTS

The three myxococcal strains utilized in this investigation of microbial predation all formed deliquescent to subspherical fruiting structures (Fig. 1) when grown on ABT agar.

When these myxococci are grown in liquid culture containing ABT they form colonial spherules (Fig. 2) as described for the PC02 strain by Burnham et al., 1981. The exact shape of these spherules depends upon the strain and its culture age. Figure 3 shows an immature colony of the BG03 strain to be almost totally comprised of swarming spikes of vegetative cells. Generally we have observed that older spherules have longer, more tufted fruiting structures. BG02 and PC02 strains form club-like semi-spherical fruiting structures on the colonial surface in liquid culture (Fig. 4). These are quite similar in organization to the fruiting structures seen in agar cultures (Fig. 1). Phase contrast microscopy of one of the small fruiting structures on an ABT plate (Fig. 5) reveals the curved rod appearance of older vegetative cells of the BG02 strain along with a cluster of myxospores. Although these strains will produce refractile myxospores as observed by phase contrast microscopy, they are more commonly seen as opaque structures (Fig. 5). Myxospores could be induced in all three strains using the glycerol technique of Dworkin and Gibson, 1964.

Figures 1-7

When grown in mixed culture with Phormidium luridum the BG02 strain will form knob like fruiting structures (arrow) on the surface of the entrapped cyanobacteria (Fig. 6). Figure 7 shows several vegetative cells and the ovoid

morphology of the myxospores present on the surface of a lytic colony of the BG02 strain. Also evident in the micrograph is the presence of the extracellular extrusions which appear to hold the entire colony together (Burnham et al., 1981).

All three myxococcal strains appear to have a wide spectrum of predatory capability with most of the cyanobacteria tested being susceptible to entrapment and lysis (Table 1). Not all cyanobacterial species are equally susceptible to these myxococci. For example, the BG02 strain can lyse the P. luridum and Nostoc muscorum species much easier than it is able to lyse the Anabaena cylindrica species. This has been determined by comparing the number of myxococci needed per ml in mixed culture to successfully entrap and lyse the prey population. Predation of N. muscorum by the BG02 strain was found to be totally independent of whether this cyanobacterium was grown in a nitrogen-free medium or in the nitrate-containing algae broth. Table 1 also lists the eukaryotic green alga, Chlorella vulgaris which we have attempted to employ as a prey population. The myxococci are very efficient at entrapping them in large clumps; however, the Chlorella are not lysed and continue to survive in the mixed culture in large colonial aggregates held together by the myxococci.

Table 1

All three myxococcal strains effectively lyse aqueous populations of P. luridum. Figure 8 illustrates that the BG02 strain is able to lyse a cyanobacterial population of 5×10^7 cells/ml more rapidly than either the PC02 or BG02 strains. All three strains, using a 1% inoculum, achieved maximum entrapment and lysis of the cyanobacteria within 3 days. The P. luridum when inoculated into AB under standard conditions grew normally.

Figure 8

Regrowth of the P. luridum following the initial predatory attack by the myxococci, i.e., culture cycling, commonly occurred and is illustrated by the turbidity curve for a BG03 mixed culture in Fig. 8 and a BG02 mixed culture in Fig. 9. In long-term experiments, such as that graphed in Fig. 9, this regrowth could occur repeatedly. Although in this particular experiment the P. luridum regrowth peaked at intervals of 11, 8 and 7 days, the mean cycle for all experiments was 8.9 days, with a standard deviation of 1.8 days. When regrowth of the prey population did occur their cell density never reached the initial levels.

Figure 9

A series of long-term serial transfer experiments were attempted utilizing M. fulvus BG02 and P. luridum. Figure 10 shows that, utilizing a serial 5% (v/v) inoculum into AB, the axenic P. luridum was able to grow back to original population levels in the 3-5 day intervals. When the myxococci were added at an initial concentration of $4x10^3$/ml the P. luridum were clumped by the myxococci and inhibited from growing. The accumulative effect of this inhibition of cyanobacterial development is to cause the gradual loss of the P. luridum from the interactive culture. We were unable to detect any P. luridum after five transfers. By dilution alone and no multiplication the expected number of P. luridum after five transfers would be $6x10^3$. In this experiment the myxococcal population increased to $1x10^5$/ml and then repeatedly achieved a level of $3x10^4$/ml through four transfers. After the fifth transfer the mycococci maintained a level of $5x10^2$/ml in spite of the undetectable levels of P. luridum.

Figure 10

A more vigorous test of the predatory capability of these myxococci was to serially transfer the mixed culture into early stationary growth phase cultures of P. luridum averaging 10^6 cells/ml. Figure 11 shows that the M. fulvus BG02 strain at 1% (v/v) inoculation levels) was repetitively able to reduce the absorbancy of the cyanobacteria. Entrapment and lysis did not occur immediately following inoculation but usually occurred after several days. Actual lysis of the P. luridum was confirmed microscopically as well as by processing the clumps in a glass tissue grinder, which then allowed a more accurate quantitation of the cyanobacterium. The P. luridum population was reduced on average of 2.7×10^3 cells/ml over 9 successive transfers. The myxococci were consistently able to multiply back to a population of about 2×10^7 cells/ml even though they were diluted 1/100 every 4 to 7 days. This experiment illustrated the stability of this predatory system, as myxococcal feeding on the P. luridum was independent of the number of transfers.

Figure 11

To investigate the minimum inoculum necessary to achieve predation of a 10^7/ml P. luridum population, a series of dilutions were carried out on a 48h M. fulvus BG02 culture. These were inoculated into similar P. luridum cultures in AB. The results, shown in Fig. 12, indicate that 0.5 cells/ml can lyse a population of 3×10^7/ml cyanobacteria, a predator to prey ratio of $1:6 \times 10^7$. Figure 12 also illustrates that as the inoculum concentration of the myxococci is reduced the lag time prior to cyanobacterial lysis increases.

The low inoculum needed for predation (Fig. 12), as well as the ability of the myxococci to maintain a low but stable population in spite of few prey cells being present, suggests that these myxococci might survive in

environemnts containing extremely low nutrient levels.

Figure 12

The predatory ability of the myxococci was not affected by the number of days they were held in AB although morphologically a significant number of vegetative cells had converted to myxospores. Although lag times prior to lysis increased to nearly a week after 50 days in AB, entrapment and lysis stil occurred. Figure 12 illustrates the multiplicative ability of the BG02 strain once it was transferred from this AB holding culture to 10^7/ml P. luridum culture. When myxospores alone were inoculated ($5x10^6$/ml) into a P. luridum culture germination occurred followed by cyanobacterial lysis.

Figure 13

DISCUSSION

This paper augments our previous description of myxococcal predation (Burnham et al., 1981) by showing that several myxococcal isolates are capable of controlling aqueous populations the the cyanobacterium Phormidium luridum over extended periods of time. The predator-prey cycling that is consistently seen in long-term experiments indicates: a) that the predatory system is not 100 percent effective in eliminating the cyanobacteria; and b) that when sufficient prey populations develop in the presence of the myxococcal colonies, they are reduced to low levels by predatory action. The predatory system was experimentally stressed by (a) serial dilutions of the interactive culture with fresh medium (Fig. 10), and (b) serial dilution of the interactive culture with moderate density populations of the prey cyanobacterium (Fig. 11). In both cases, the myxococci responded to controlling the amount of cyanobacterial growth and reducing the overall

population of cyanobacteria present.

Because the myxococcal population increased with each successive decrease in the cyanobacteria, in the complete absence of any heterotrophic nutrient, nutrient had to transfer from the cyanobacteria to the predator. Algal to bacterial nutrient exchange is not unusual in such interrelationships; however, these are usually symbiotic or commensal in that they occur without penalty to the algal or cyanobacterial partner (Ward and Moyer, 1966; Lange, 1971). The lytic or enzymatic nature of the myxococci (Sudo and Dworkin, 1972) upsets this normal balance by damaging the cell integrity of the cyanobacterium.

The host susceptibility spectrum of this predatory mechanism appears very dependent upon the individual myxococcal strain. From the data presented in Table 1, the BG02 strain shows the broadest predatory ability, with 10 cyanobacterial species lysed. Many of the negative hosts are effectively entrapped by the myxococci but not significantly lysed. This same situation is seen with the green alga, Chlorella vulgaris. Daft et al. (1975) showed that cyanobacteria-lysing bacteria are very common in fresh water habitats and that these were capable of lysing many species of bloom-forming cyanobacteria. Four previous myxobacterial isolates, later identified as Lysobacter sp. (Christensen and Cook, 1978), were studied in detail and, although they all showed broad lytic ability, each isolate possessed a slightly different spectrum of lytic capability, similar to that shown for the three myxococcal strains in this paper.

The myxococci were able to control the P. luridum populations with inocula as low as 50 cells/100ml. This is in marked contrast to the requirements of the Lysobacter CP-1 studied by Daft et al. (1975) which needed inoculum levels of 10^6/ml for successful lysis the low population levels of the BG02 strain. The required level of M. fulvus BG02 however, was similar to the levels of myxobacteria found by Daft et al. (1975) to be present in natural surface

waters, i.e., from 1 to more than 110/ml with a mean of 44/ml for seven different habitats. The development of predatory colonies from such low inocula could be explained by their use of cyanobacterial secretions as early nutrient sources (Daft et al., 1975). Ward and Moyer (1966) demonstrated such potential by showing that the growth of non-predatory bacteria in mixed culture in an autotrophic medium paralleled the growth of the algal partner.

In our initial description of the morphology of the predatory colony we suspected the processes of swarming and spore formation to be important mechanisms in the entrapment and lysis of the cyanobacterial cells (Burnham et al., 1981) by these lytic colonies. The slight differences that these three myxococcal strains show with respect to the formation in liquid medium of 1) lytic colonies, and 2) fruiting structures, appear to have no effect on their ability to entrap or lyse the P. luridum. The formation of aggregates of the myxobacterium CP-1, now Lysobacter, was reported by Daft and Stewart (1973). In addition, when they added high numbers of Lysobacter to blooms of cyanobacteria the viable counts of the lytic bacteria fell rapidly. Such a drop would be expected when the myxobacteria aggregate into colonial units if fragmentation techniques were not employed in the myxobacterial enumeration.

This aqueous colonial morphology of the myxococci suggests an interesting analogy with the predatory bacteria, the bdellovibrios. These predators penetrate the host bacterium's cell wall and enjoy the environmentally-protected periplasm in which to enzymatically liberate prey nutrients (Rittenberg and Thomashow, 1979). This is very similar to the protected core created by the peripheral myxococcal vegetative cells in the colony. The massive encapsulating myxococcal population provides, through its collective swarming activity and fibrous adhesions (Burnham et al., 1981), an analogous mechanism to the Gram-negative wall of the infected bdellovibrio host. Huffaker et al. (1976) contend that predators need to possess several attributes to be successful control agents: adaptability to physical

conditions; searching (or trapping) capacity; ability to multiply; power of prey consumption; and ability to survive in periods of low host density. The description presented earlier by us (Burnham et al., 1981) on the nature of the aqueous myxococcal predatory colonies, coupled with the structural and population data described in this report indicate that the myxococcal predatory system tested in vitro does possess such attributes. Such abilities should ensure and explain the survival of the myxococcal species in fresh water habitats.

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Table 1

Comparative Cyanobacterial Susceptibility to Aqueous Myxococcal Predation

	Myxococcal Predator Strain		
Tested Prey Species	PCO2	BGO2	BGO3
Anabaena cylindrica[1] UTEX B629	+/-	+	-
Anabaena variabilis UTEX 377	+	-	-
Aphanizomenon flos-aquae[2]	ND	+	-
Lyngbya sp. UTEX 622	+/-	-	-
Microcystis aeruginosa[2]	ND	+	ND
Nostoc muscorum[3]	-	+	+
Oscillatoria sp. CBS 15-1865	-	-	-
Phormidium faveolaum UTEX 427	+	+	+
Phormidium luridum (UTEX 426)	+	+	+°
Plectonema boryanum ATCC 18200	+	+	+
Symploca muscorum UTEX B617	-	-	-
Synechococcus sp. (Stanier 6908 strain) (previously ATCC 27146)	+	+	+
(*Chlorella vulgaris*) UTEX 260	-	-	-

[1]University of Texas Culture Collection of Algae

[2]Harvested directly from natural blooms (Balgavies Reservoir and Long Loch, Scotland)

[3]Department of Biological Sciences, U. Dundee, Dundee, Scotland

ND = Not done

LEGEND FOR FIGURES

Fig. 1 Myxococcus xanthus BG03 fruiting structure after incubation for 7 days at 23°C on ABT agar. Bar equals 600 um.

Fig. 2. Light microscopy of a mature colony of M. xanthus BG03 grown for 8 days on ABT broth. Bar equals 400 um.

Fig. 3. Light microscopy of an immature colony of M. xanthus BG03 grown for 4 days on ABT broth. Bar equals 125 um.

Fig. 4. Light microscopy of the fruiting body structures on the surface of colonial spherule of M. fulvus BG02 for 7 days in ABT. Bar equals 100 um.

Fig. 5. Phase contrast micrograph of a segment of a fruiting body formed by M. fulvus BG02 after 7 days of growth on an ABT agar plate. Bar equals 20 um.

Fig. 6. Light microscopy of a predatory colony of M. fulvus BG02 after growth for 5 days in a culture of Phormidium luridum in AB medium. Note the presence of knob-like fruiting structures (arrows) on the colony surface. Bar equals 500 um.

Fig. 7. SEM of the myxospores and rod-shaped vegetative cells present on the surface of a fruiting structures, on a predatory colony. Bar equals 3 um.

Fig. 8. Comparative clearing ability of the myxococci (10^6 cells/ml at 0 days) on a population of 10^7 Phormidium luridum cells/ml at 25°C. Myxococcal strains: BG02 (●); BG03 (▼); and PC02 (■). P. luridum control in AB (○).

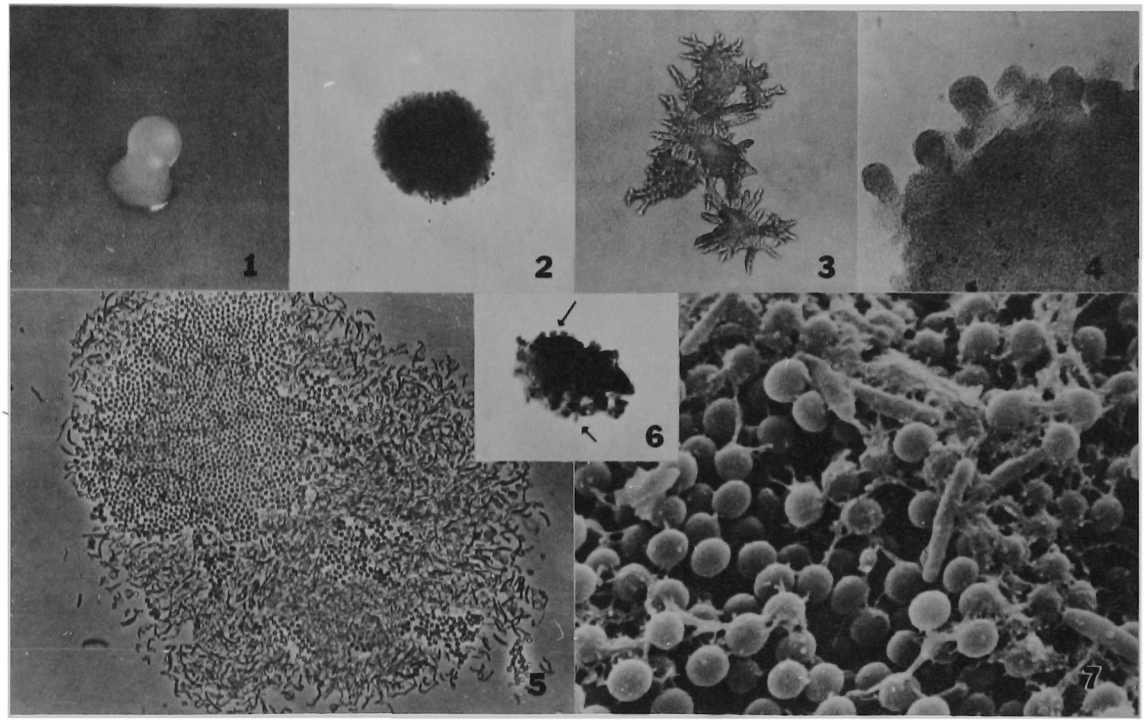
Fig. 9. Cyanobacterial density fluctuations observed when Myxococcus fulvus BG02 (10^5/ml at 0 days) is incubated with P. luridum (10^7/ml at 0 days) at 25°C.

Fig. 10. Effect on P. luridum population levels of serial 5% transfers into fresh AB: Axenic P. luridum (·····); P. luridum and M. fulvus BG02 (10^5/ml at 0 days) (—·—·—). Vertical lines represent transfer points.

Fig. 11. Effect on P. luridum density of serial 1% transfers into mature P. luridum cultures (between 10^5 - 10^7 cells/ml). The initial culture contained approximately 10^8 P. luridum/ml and 10^5 M. fulvus/ml. Vertical dashed lines represent transfer points.

Fig. 12. The effect of different initial M. fulvus BG02 concentrations on the ability to equal constant ($3x10^7$/ml) populations of P. luridum. Myxococci/ml: $5x10^2$ (●); $5x10^1$ (■); $5x10^0$ (□); $5x10^{-1}$ (○); $5x10^{-2}$ (◇).

Fig. 13. Population changes of M. fulvus BG02 upon inoculation into constant populations of P. luridum (10^7 cells/ml). The myxococci were maintained in AB at 25°C for the number of days indicated by the lower point of each slope prior to being transferred to the cyanobacterial culture.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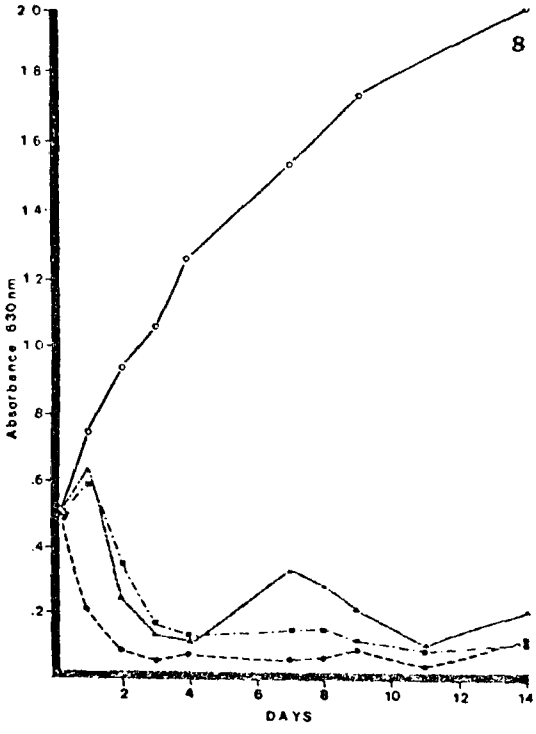
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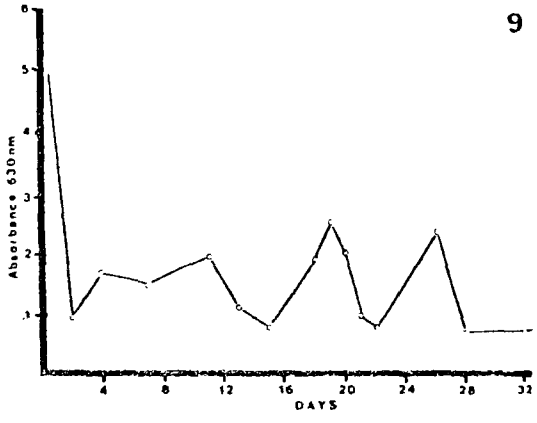
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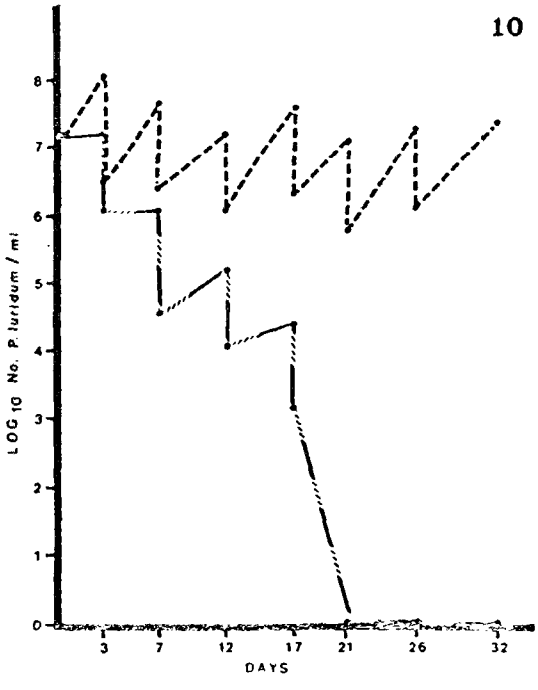
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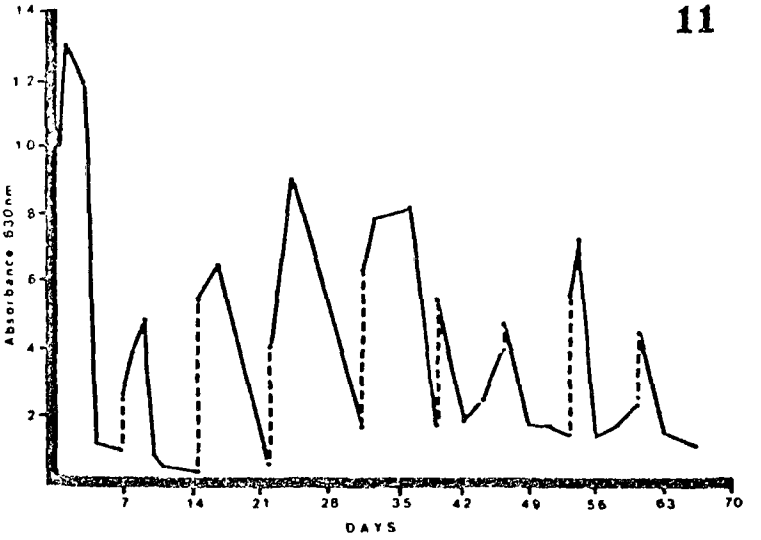
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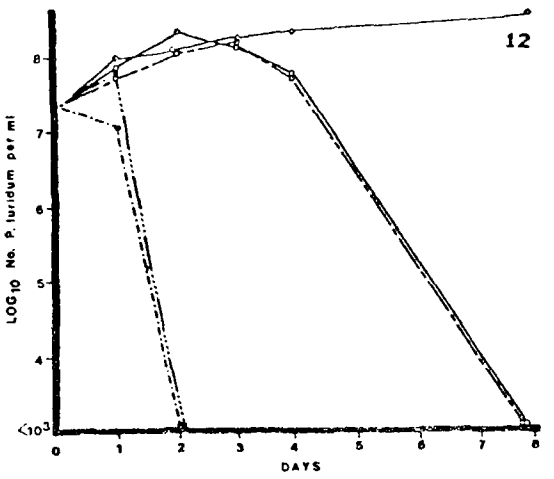
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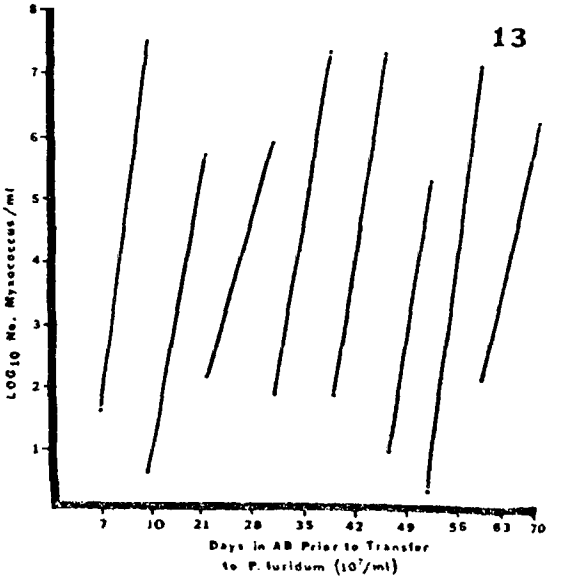
13


LOG10 No. Mycococcus/ml
Days in AB Prior to Transfer
to P. luridum (10⁷/ml)

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Lysis of *Phormidium luridum* by *Myxococcus fulvus* in continuous flow culture

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Summary

In two chemostat systems *Myxococcus fulvus* (strain BG02) grew attached to either a) the glass walls of the growth vessel producing long colonial extensions toward the centre of the pot, or b) glass beads organized in a 68 cm vertical column. Both systems enabled measurement of lytic enzyme production and cyanobacterial predatory efficiency. Lysozyme activity produced by the myxococci was dependent on the concentration of the tryptone in the medium and its flow rate. Continuous lysis of *Phormidium luridum* occurred in both chemostat and glass bead culture in the presence of BG02. The data suggests that adherence characteristics of this species prevents the achievement of steady state kinetics in either saprophytic or parasitic modes of growth.

M. fulvus with its various morphological growth forms and effectiveness in lysing cyanobacteria is considered to be a potential control agent.

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INTRODUCTION

The complex biological interactions within cyanophycean blooms have been discussed by Stewart & Daft (1977). Lysis of cyanobacteria by bacteria may be brought about by the production of extracellular products (Stewart & Brown, 1971), a contact mechanism (Shilo, 1970; Daft & Stewart, 1973) or by entrapment (Burnham *et al.*, 1981). Actinomycetes (Daft *et al.*, 1983) may produce active extracellular products capable of killing a wide variety of microorganisms. Perhaps the most numerous and wide spread agents capable of destroying cyanobacteria are the free living amoebae. They are found in most fresh waters, so far examined, that contain cyanobacteria (Yamamoto & Daft, 1983) and in mires (Yamamoto & Daft, 1983). Some of the attributes suggested by Huffaker *et al.* (1976), i.e., adaptability to physical conditions, searching (or tappping) capacity, ability to multiply, prey consumption and survival in periods of low host density, are possessed by each of these agents that have the ability to kill cyanobacteria. Myxococci, first shown by Burnham *et al.* (1981) to entrap and then lyse *Phormidium luridum*, probably come close to the ideal control organism desribed so far. Recently *Myxococcus fulvus* strain BG02 has been shown to sustain lysis of *P. luridum* at allow levels and to survive periods of low prey densities (Burnham *et al.*, 1983).

The production of spherules by these myxococci (Burnham *et al.*, 1981) and the necessity for the integrity of the spherules for lysis (Burnham *et al.*, 1983) produces some practical problems in their large scale cultivation. This paper describes attempts at culturing *M. fulvus* alone and during its lysis of *P. luridum* in a continuous flow culture system.

MATERIALS AND METHODS

Organisms:

Myxococcus fulvus, strain BG02 (Burnham *et al.*, 1981) was maintained on Algae Broth (Difco) medium containing 0.2% Bactotryptone (Difco) and designated ABT medium. *Phormidium luridum* var *olivacea* (No 426 from the University of Texas) was kept in pure culture in a similar medium without tryptone (AB).

Cultural Techniques:

M. fulvus and *P. luridum* when grown in liquid culture both singly and together were kept on a rotary shaker (100RPM) under a photon flux density of 100 u mol m^{-2} s^{-1} at 26°C. Routinely the myxococci were subcultured by douncing spherules in a glass homogenizer then adding the cell suspension to fresh ABT medium. For inoculation into mixed cultures the *M. fulvus* was centrifuged from ABT cultures, washed twice in AB medium then homogenised in AB medium with a glass tissue grinder (Wheaton) and finally added to the *P. luridum* culture in AB medium. Routine cultures also were maintained in 500 ml conical flasks each containing 100 ml of medium.

Continuous flow cultures for *M. fulvus* BG02 were set up in two configurations:

1) System 1 utilized a magnetically stirred 1200 ml vessel containing 750 ml of medium controlled with a single overflow located at the liquid surface (see Fig. 1). The fresh medium was pumped in at either 7.0, 10.0 or 40.0 ml/hr. Air was bubbled in at 1.0 l min^{-1} and the system was maintained at 23°C.

2) System 2 utilized the surface of glass-beads (4 mm diam) contained in a glass tube, 68 cm long x 0.9 cm internal diameter. Each tube contained approximately 650 glass beads. The liquid media was supplied at 5 ml h^{-1} to the tubes either at the top or at the bottom depending on the particular experiment. The tubes had 21.5 cm^3 of medium volume, 21.8 cm^3 volume of glass beads with a total surface area of 519 cm^2 (327 from the beads and 192 cm^2 from the internal surface of the tube). Aeration and sampling procedures are given in the Results section. Inoculation of the tubes was accomplshed by injecting 3 ml of a homogenized BG02 culture 10^6 cells ml^{-1}) through the rubber bung containing the medium inlet.

P. luridum, when required for experiments in which it was added continuously to the culture vessel containing M. fulvus was grown in 8 l of AB medium in glass vessels, aerated, stirred and under the light regime described above.

Quantitative Measurements

Lysozyme activity was measured by the reduction in turbidity at 540 nm of Micrococcus lysodeikticus (lysozyme substrate, ex Difco). To 1 ml of the substrate in phosphate buffer (pH 7.2) was added 4 to 1 ml of the buffer plus 5 to 8 ml of the filtered sample to give a final reaction mixture of 10 ml. the difference in optical density before and after incubation for 30 min at 37°C multiplied by 10^3 gave the enzyme activity.

Protein levels in the bacteria free samples were estimated by two methods: the absorption at 280 and 260 nm (Herbert et al., 1971) and by the method of Lowry, et al. (1951).

Viable bacterial counts or colony forming units (CFU) were made on spread plates containing ABT medium. The samples were dounced 25 times in a glass

homogeniser (Wheaton) then diluted in ABT medium. This method was used for estimating inocula and sample colony counts.

Chlorophyll levels were estimated by the method of Parsons and Strickland (1963).

The bacterial pigment was extracted in hot methanol, centrifuged (1,000 rpm for 15 min) and the supernatant scanned over the range of 325-550 nm.

Absorption was read either in an MSE Spectro Plus or in a Unicam SP 700 spectrophotometer.

Microscopy

Myxococcal colonial formations were gently removed from the growth flask and immediately fixed in 0.1M KH_2PO_4 (pH 7.2) buffered glutaraldehyde (4%). for light microscopy cells were examined and photo graphed using a Zeiss Photomicroscope III. For scanning electron microscopy (SEM) glutarladehyde fixed cells were subsequently dehydrated and critically point dried in baskets and then applied toaluminum stubs using double-sided tape. All specimens were coated with gold-palladium and then examined with a JEOL 35 scanning electron microscope.

RESULTS

Continuous Culture of M. fulvus in System 1

Although some growth can be seen on the surface of the glass growth vessel (Fig. 1) this is better illustrated in the photograph in Fig. 2. The M. fulvus BG02 initially tended to adhere to the walls of the growth vessel and then long strands of the bacterium appeared to radiate toward the center of the vessel. These strands often measured 5 cm in length and could become 0.5 cm wide. Figure 3 shows an example of the basal growth found along the bottom of the vessel with fruiting structures (arrows) visible along the inner surface (medium side) of the colony structure. Light microscopy of the

"feathery" ends of these strands (Fig. 4) show an intricate array of the myxococci, providing enormous surface area for nutrient exposure. Higher magnification of the ends of these strands (Fig. 5) shows the rod-shaped vegetative cells are held together by numerous fibers. No fruiting structures appear associated with this region.

Observation of the basal growth attached to the wall of the growth vessel (Fig. 6) in sheets shows the orientation of fruiting structures along raised ridges on the inner or medium surface of the colony. Higher magnification (Fig. 7) illustrates the commonly observed raised fruiting structures formed by the swarming activity of the rod-shaped vegetative cells. The height of the stalk appears variable but all of the heads of these fruiting bodies show the presence of myxospores which have been described previously (Burnham et al., 1981; 1984).

This type of growth resulted in the overflow being non-homogeneous. In the first few days of culture, semi-spherical colonies of the type described previously (Burnham et al., 1981) were found in the effluent. Meaningful viable cell counts in the overflow were not obtainable because very few unattached BG02 cells could be detected in this overflow from the growth vessel. Later strands would occasionally break loose and wash out. As a result a steady state of the culture was never reached. After about 20 days, the strands would usually lose their ability to adhere to the glass walls of the vessel and the whole culture would then slowly wash out.

Figure 8 presents the lysozyme activities that were measured in the effluent of continuous cultures containing M. fulvus grown in ABT medium supplied at the rates of 40 and 10 ml h^{-1}. The initial rise in activity was faster at 10 ml h^{-1} and a plateau was reached after 3d and lasted for 9d when there was a slow decline in actiivty. At the faster rate of medium addition

maximum activity was reached after 9d and this was approximately 3 times the maximum for 10 ml h^{-1}. This high activity could only be maintained for 2d before a rapid decline in activity was observed. The pH of the effluent from both cultures increased from 7.2 to 8.1 units and protein levels showed little change from lg l^{-1} over the experimental period.

Lysis of P. luridum by BG02 in Continuous Culture (System 1)

The efficiency of BG02 in continuous culture to lyse P. luridum was tested by first culturing the bacterium in ABT medium at rates of 7 and 40 ml h^{-1} for 5 days and then adding an actively growing culture of P. luridum at the same two rates. Data for lysozyme activity and entrapment of the cyanobacterium are given in Table 1. Both rates of ABT medium addition gave maximum lysozyme activities after 5 days. The 40 ml d^{-1} application produced the higher activity. When the P. luridum culture was substituted for the ABT medium lysozyme activities in each vessel dropped rapidly over the following 3 days and then more gradually over the next 4 days finally reaching approximately similar values. The P. luridum culture in the reservoir continued to grow and this is seen in the total amount of chlorophyll a supplied to each vessel per day. At each rate of addition the amount of chlorophyll a entrapped by the bacterium increased. Taking into account the increasing quantity of cyanobacteria added per day, the efficiencies of entrapment declined as the experiment progressed and this decline was more marked at the high flow rate. Final readings taken after 17d gave efficiencies of 41 and 13% for the 7 and 40 ml d^{-1} flow rates respectively.

Culture of BG02 on Glass Beads (System 2)

Because isolate BG02 has the strong tendency to adhere to glass surfaces a simple apparatus was used to exploit this adherence phenomenon. Three modifications of this apparatus were used and these are shown in Fig. 9a, b, and c. The first experiments compared the culturing of BG02 in flasks with that in the column. Two levels of ABT medium, normal (ABT/1) and 1/10 concentration (ABT/10) were used, 100 ml contained in the 500 ml flasks and a rate of 5 ml h^{-1} flowed into the column (Fig. 9a). Figure 10 shows the type of growth that occurred on and between the individual beads comprising the upper third of the column. The amount of adherent growth decreased in the middle and lower thirds of the column. As the culture aged, fruiting structures formed identical to those seen in Figures 6 and 7. Lysozyme (Fig. 11a) activity was higher in both methods of culture when BG02 was supplied with undiluted ABT medium. Both ABT/1 and ABT/10 media resulted in reaching a maximum level of lysozyme activity at 13 days from inoculation. Protein content of the effluent from the column given ABT/1 medium fell by 44% after 3d, then remained relatively constant. The drop in protein content was much more marked in the corresponding flask culture (Fig. 11b). At the lower medium concentration in both culture methods there was a very gradual reduction in protein content throughout the experimental period. Viable cell counts in the flask culture containing ABT/1 medium declined uniformly from 10^6 to 80 CFU ml^{-1} over the 24d period. From inoculation, cell counts in the column effluent fell from 10^6 to 0 and remained at 0 CFU for 5d and then increased to a maximum after 13d and remained at this value (Fig. 11c). The pH values in all four cultural systems increased from an initial value of 6.8 to a final value of 7.7 units. When the experiment was terminated the glass beads were removed and 10 representative beads from the top, middle and lower

segments were extracted in methanol. After centrifugation (7×10^3 rpm) the supernatant was scanned over the range of 320 to 550 nm. The absorption curves for each segment are given in Fig. 12. Comparatively, taking the maximum absorption of the upper third as 100 the middle and lower segments had values of 50 and 22 respectively. This indicates that approximately 60% of the growth of BG02 took place on the top segment nearest to the medium input and only 30% and 10% in the middle and lower segments respectively.

Lysis of P. luridum by BG02 Grown on Glass Beads (System 2)

The second modification of the column glass beads involved aerating from the bottom to give a counter-flow with regard to the medium (Fig. 9b). Samples were taken daily from the flask (S) kept at the head of the column. ABT/1 medium was added to the column 5 ml h^{-1}) for 20d. The medium feed was then changed to an actively growing culture of P. luridum in AB. The lysozyme activity reached a maximum after 20d but then rapidly declined to zero activity after 32d. This decline corresponded to the addition of the P. luridum culture. A drop in protein content from 1.8 to 1.0 ug ml^{-1} occurred during the first 20d, then after addition of the P. luridum culture, a consistent levels 0.1 ug ml^{-1} was reached within 2d. The protein levels in the P. luridum reservoir stayed fairly constant, at around 0.03 ug ml^{-1} for the succeeding 14d. Figure 13 shows the absorbance data from this experiment to determine the lytic efficiency of the M. fulvus within the column in lysing the cyanobacteria. The reservoir containing the P. luridum showed an increase in absorbance from 0.10 to 0.21 over the 12d period due to the growth of the cyanobacterium. The cyanobacterial content of the effluent from a glass bead control column (without M. fulvus) was very nearly equal to that of the reservoir and closely paralleled it throughout the experiment. The effluent absorption at 630 nm from the BG02 containing column increased to just over

50% of the influent (reservoir). These data indicate that 1) the M. fulvus BG02 was still active in trapping and lysing the cyanobacterium after 12d; 2) that 64% of the cyanobacteria had been removed from the influent during the course of the experiment; and 3) the presence of the M. fulvus increased the trapping efficiency of the plain beads by over 6-fold.

The third modification introduced both air and medium into the bottom of the column and effluent of the growth column construction was collected from the top (Fig. 9c). Good growth of BG02 was produced after only 3d and then the ABT/1 medium was changed to the P. luridum culture in AB medium. Chlorophyll contents of the reservoir and the effluent from the column are given in Table 2. This third method of culturing the BG02 gave a very heavy growth of the BG02 strain within 3 days from inoculation and the rate of entrapping and lysing of the Phormidium was very efficient. Chlorophyll a contents of the reservoir remained at an average level of 2.2 mg/l^{-1} throughout the 8d period during which the Phormidium was added. In the effluent from the M. fulvus column containing the maximum value of chlorophyll a was only 0.75 mg/l^{-1} during the same period. After 2d the effluent from the column contained 10% of the total chlorophyll a content applied and even after 9d this had only risen to 30% of the input.

DISCUSSION

M. fulvus can exist in several different morphological forms. When grown in flask culture it produces spherules (Burnham et al., 1981) which are effective in lysing cyanobacteria at both high and low concentrations (Burnham et al., 1984). However, it has been shown in this investigation that it has a strong tendency in continuous culture to adhere to the glass walls of the growth vessel or to glass beads. Although colonial spherules formed early in the vessel culture they washed out at a rate greater than their formation and

were not a significant growth form in long term culture. It appears that M. fulvus BG02 is effective in lysing cyanobacteria in either the free floating spherule form or in the adhered colony. The coated glass beads make use of the adherent ability of the bacterium and in this static position it efficiently entraps the cyanobacterium as the culture is pumped through the column. As only approximately 30% of the total chlorophyll a supplied from the reservoir was found in the effluent of either of the columns shown in 9b or 9c this would appear to be an efficient technique for bringing about lysis.

In each of these three culture configurations the BG02 is difficult to enumerate. The colonial spherules can be dounced in a glass homogenizer and then plated out after dilution, but it is more difficult to obtain meaningful results when the myxococci are growing attached to a glass surface. However, using indirect techniques, it was possible to compare growth on beads and in culture flasks (Fig.10). The protein levels and the number of cells from the effluent in the ABT/1 treatment suggests that beads give a better yield of BG02. At the end of this experiment the beads from the proximal third of the growth column were heavily coated with BG02 while the distal end contained few myxococci. This appears very analogous to the cross-sectional structure of the colonial spherule from the outside of the core. Maintenance of the column as an effective lysing system would require more data on both flow rates and the concentration of the cyanobacterium. Burnham et al. (1983) have shown that BG02 in spherule form can maintain its predatory capability over a long period (greater than 70 days) and at low inoculation levels. In continuous culture, the multiplication of algophorus amoebae and their rates of predation are influenced by the supply of the prey (Yamamoto and Daft, 1983). Particularly in the vessel system we suspect that the survival of the system, ie., retention of adherent ability to glass, is dependent similarly to the

availability of nutrient, either cyanobacterial prey or the ABT medium, to the underlying adhering cells. If this is blocked by overgrowing vegetative cells then release and subsequent washout occurs. The gross filtering capability of the glass bead column seems to prevent this allowing reseeding to occur.

Isolate BG02 was originally isolated from a drainage ditch alongside an agricultural field. It is possible that all three morphological colonial forms of this myxococcal species occur naturally in this sort of environment. The spherules, being free, move with the currents of the drainage water, the feathery form may adhere to higher plant organs growing in the ditch and small stones could become coated and act as an anchorage. In each form the bacterium would be able to entrap suitable prokaryotes growing in this aqueous environment.

In a previous paper (Burnham et al., 1984) we showed that myxospores of M. fulvus could initiate lysis of a cyanobacterium, that the vegetative cells could survive in a non-nutrient environment and that active lysis could be maintained over a long period. In this present work the Myxococcus BG02, by its range of growth forms, has shown its ability to adapt to two flowing systems and in both cause significant cyanobacterial lysis.

ACKNOWLEDGEMENT

Appreciation is expressed to Francis for technical assistance. This investigation was supported in part by Grant B-086-OHIO from the Ohio Water Resources Center and the Office of Water Resources and Technology, U. S. Department of Interior.

Legend for Figures

Figure 1. Photograph of System 1 growth vessel containing 750 ml of ABT medium controlled by outlet at right. Inlets for medium addition and aeration are at top. The entire vessel is positioned on a magnetic sitrrer for agitation.

Figure 2. Photograph of colonial strands (arrows) formed by M. fulvus BG02 in System 1. Bar equals 10 mm.

Figure 3. Darkfield photomicrograph of 12-day basal growth of M. fulvus BG02 along bottom of the System 1 vessel. In addition to the strands forming at the periphery of the colony numerous fruiting structures (arrows) can be seen on the colonial surfaces. Bar equals 1.0 mm.

Figure 4. Phase contrast photomicrograph of the periphery of an M. fulvus BG02 colony growing in the System 1 vessel after 9 days of culture. Bar equals 100 um.

Figure 5. SEM of the tip of a strand from a colony from the above culture. Note the network of fibers (arrows) appearing to hold the rod-shaped cells of M. fulvus BG02 together. Bar equals 1.0 um.

Figure 6. SEM of M. fulvus BG02 growth along glass surface at 12 days showing surface knob-like fruiting structures (arrows). Bar equals 100 um.

Figure 7. SEM of fruiting structure located on basal growth described in Fig. 6. Bar equals 10 um.

Figure 8. Lysozyme activity (see Methods) contained in the system 1 growth vessel effluent from continuous cultures maintained at 10 ml ABT h^{-1} (X) and 40 ml ABT h^{-1} (●).

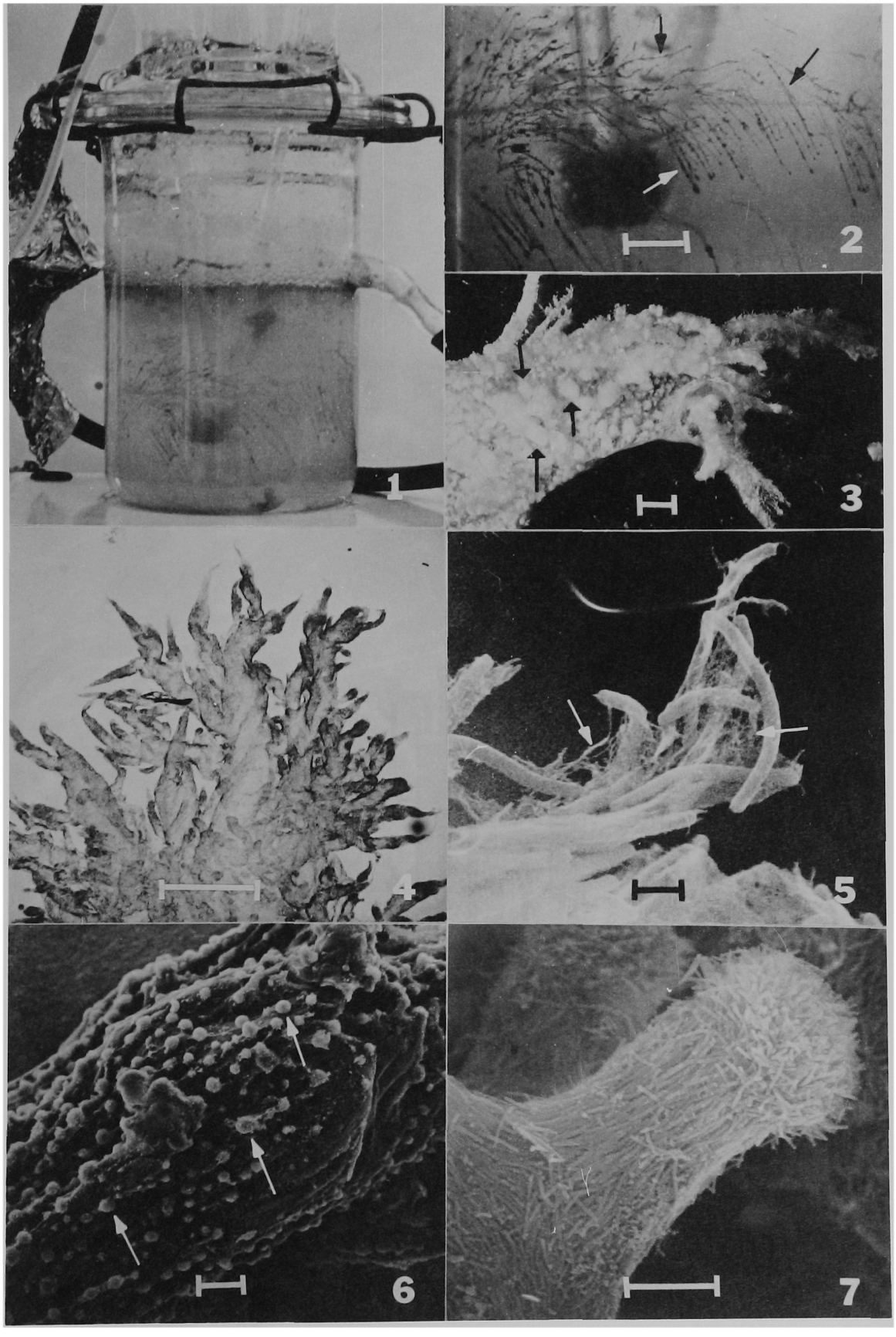
Figure 9. Diagrams of three modifications of a glass bead filled vertical glass column (System 2) used for continuous culture of M. fulvus BG02. AP, air pump; F, filter; BG; 4.0 mm dia. glass beads; PP, peristaltic pump; S, sampling site; V, filtered vent; T, 68 cm, 0.9 cm ID glass tube.

Figure 10. Photograph of the 14 day adherent growth of M. fulvus BG02 in the upper third of the glass bead filled column maintained at a flow rate of 5ml ABT h^{-1}.

Figure 11. Lysozyme activity (a), protein content (b) and viable cell counts contained in M. fulvus culture effluent. Triangles, continuous flow culture column as in Fig. 9a; circles, flask cultures; solid symbols, ABT/10 medium; open symbols, ABT/1.

Figure 12. Comparative pigment extraction from M. fulvus BG02 adhered to 10 glass beads taken from the upper (U), middle (M) and lower (L) third of a continuous culture column (Fig. 9a) maintained at 5 ml ABT h^{-1} for 24 days.

Figure 13. Effect of M. fulvus contained in a glass bead filled column (Fig. 9b) on clearing P. luridum from a 5 ml h^{-1} influent. O, influent (P. luridum in AB medium from growth reservoir); ●, effluent from uninoculated glass bead control column; □, effluent from M. fulvus containing column.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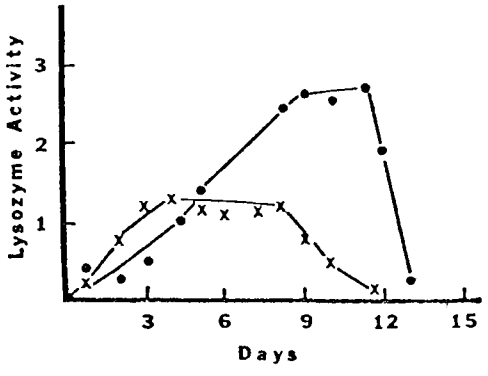

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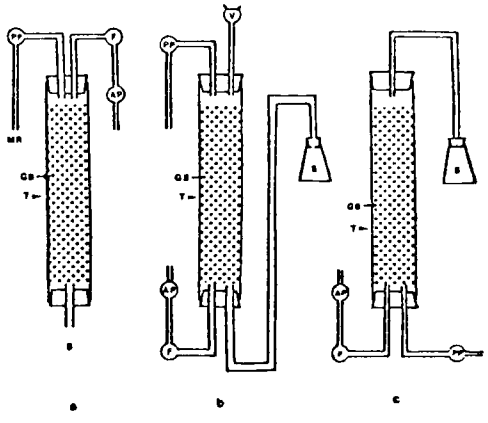

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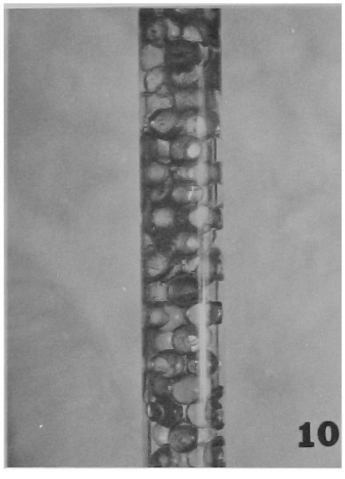
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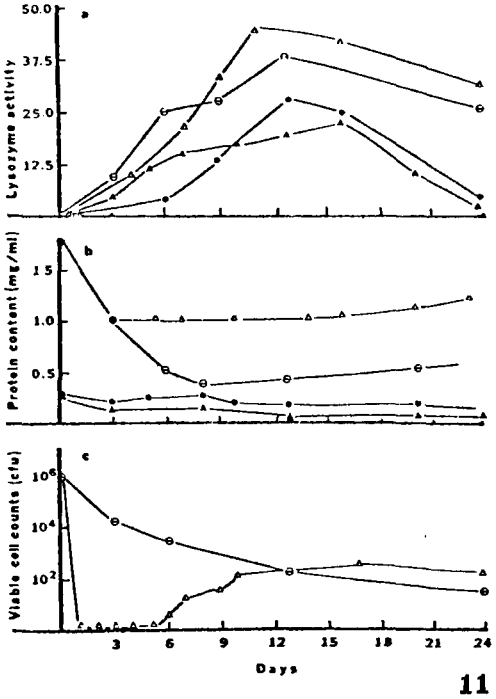
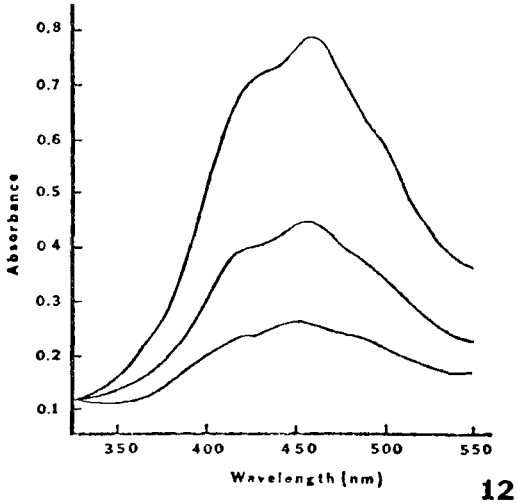
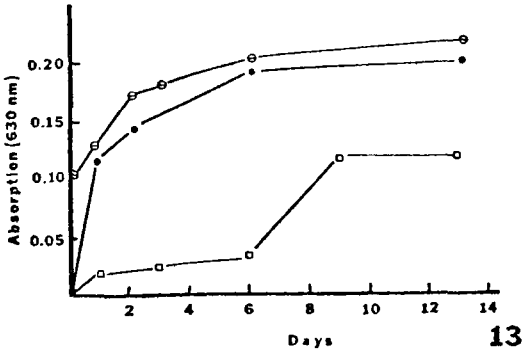

a
Lysozyme activity
50.0
37.5
25.0
12.5
b
Protein content (mg/ml)
1.5
1.0
0.5
c
Viable cell counts (cfu)
10^6
10^4
10^2
3
6
9
12
15
18
21
24
Days

11


Absorbance
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
350
400
450
500
550
Wavelength (nm)

12


Absorption (630 nm)
0.20
0.15
0.10
0.05
2
4
6
8
10
12
14
Days

13

TABLE 1: Lysozyme activities and amounts of *Phormidium luridum* entrapped by *Myxococcus fulvus* grown in chemostats at 2 flow rates. Cultures supplied with ABT medium for 5 days then with *P. luridum* in AB medium.

		Days from inoculation											
	Flow rate	1	2	3	4	5	6	7	8	9	10	11	12
		ABT medium					*P. luridum* in AB supplied continuously						
Lysozyme activity	(7ml h^{-1})	0.25	0.15	0.60	0.75	2.35	2.00	1.80	1.25	1.50	1.10	1.00	0.90
	(40ml h^{-1})	0.30	0.35	1.25	2.20	3.40	2.80	1.00	1.02	0.90	0.75	0.70	0.70
Total amount of Chl. *a* applied mg d^{-1}	(7ml h^{-1}						0.54	0.58	0.63	0.70	0.77	0.81	1.03
	(40ml h^{-1}						3.10	3.37	3.64	4.04	4.45	4.66	5.96
Total amount of chl. *a* entrapped mg d^{-1}	(7ml h^{-1}						0.47	0.51	0.51	0.56	0.61	0.50	0.70
	(40ml h^{-1}						2.65	2.67	2.70	2.91	3.07	3.00	3.32
Entrapment efficiency d^{-1} (%)	(7mlh^{-1}						87	88	81	80	79	62	66
	(40ml h^{-1}						86	79	74	72	70	64	57

TABLE 2: Lysis of *Phormidium luridum* by *Myxococcus fulvus* (BG02) grown on glass beads within a column. *M. fulvus* grown with ABT medium (5 ml h^{-1}) for 3 days then supplied with a culture of *P. luridum* at 5 ml h^{-1}

		Days from inoculation											
	Flow rate	1	2	3	4	5	6	7	8	9	10	11	12
		ABT medium			*P. luridum* in AB supplied continuously								
Chl. *a* content ($mg\ l^{-1}$) of *P. luridum*	Reservoir	-	-	1.97	2.01	2.15	2.57	2.35	2.10	2.23	2.35	2.40	2.20
	Column effluent	0	0	0	0.05	0.21	0.27	0.36	0.37	0.47	0.45	0.55	0.75

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